Renal Structural Changes after Kidney Allograft Transplantation

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Renal Structural Changes after Kidney Allograft Transplantation

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Contents

Chapter 1

Introduction and outline of the thesis

Introduction

Late transplant dysfunction and transplant loss remains an important problem after kidney allograft transplantation.[1] The prospects of a patient who falls back to dialysis treatment after kidney transplantation are bad; not only is the quality of life severely affected but also survival is poor.[2] A gradual decline in kidney allograft function is observed in approximately 40 to 50% of the patients two or more years after transplantation.[3-4] The loss of function is often accompanied by an increase in blood pressure and proteinuria. Various factors, alloantigen-dependent and alloantigen-independent, may contribute to this gradual loss of function, a syndrome which has been designated chronic transplant dysfunction (CTD) (Figure 1). When CTD is recognized, a biopsy is usually taken after prerenal (arterial obstruction) or postrenal (urine tract obstruction) causes of functional decline are excluded. The biopsy sample that has been taken may demonstrate a specific cause of dysfunction such as recurrent glomerular disease or a *de novo* glomerulopathy, however, in the majority of cases non-specific chronic changes are found that do not allow exact identification of the cause(s) of graft decline.[5-9]

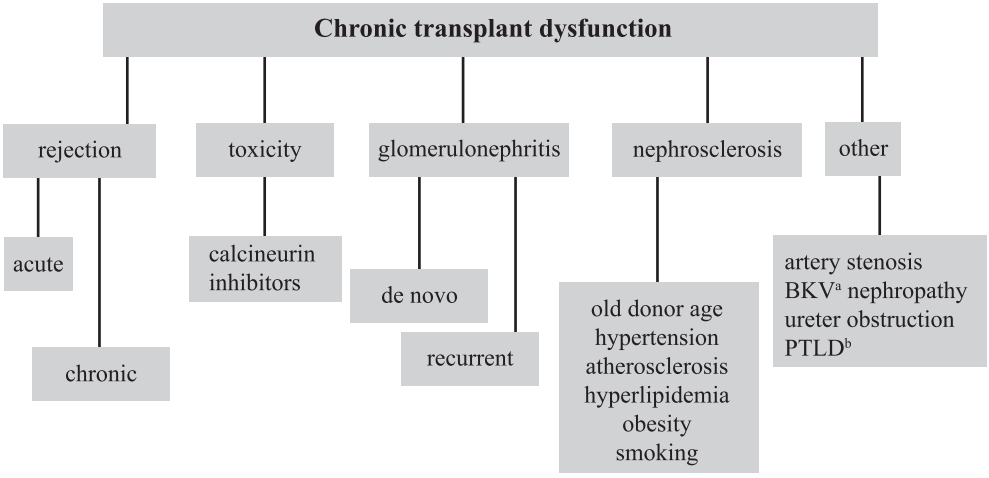

Figure 1: Factors that may cause chronic transplant dysfunction.

[a] BKV, polyomavirus type BK en [b] PTLD, post-transplant lymphoproliferative disease.

Since 1991 efforts have been made to standardize the interpretation of pathologic findings of renal allograft biopsies in the Banff Working Classification of Renal Allograft Pathology. This system uses the term chronic allograft nephropathy (CAN) (Table 1) to classify the chronic/sclerosing changes, which include chronic obliterative vascular alterations, tubular atrophy, glomerulosclerosis, and interstitial fibrosis.[10-11] CAN is graded by the severity of interstitial fibrosis and tubular atrophy because they are most accurately assessed, develop regardless of the etiology of allograft decline, and correlate with the degree of functional impairment. [12] Interstitial fibrosis is considered to be present when the supporting connective tissue in the renal parenchyma exceeds 5% of the cortical area.[10] Tubular atrophy refers to the presence of tubules with thick redundant basement membranes, or a reduction of greater than 50% in tubular diameter compared to surrounding non-atrophic tubules.

The causes of CAN are multifactorial (Figure 2) and include both chronic rejection and chronic calcineurin inhibitor toxicity.[11] A recently published large study of protocol biopsies of patients who received a kidney-pancreas transplant identified two distinctive phases of tissue injury leading to CAN. An early phase with tubulointerstitial damage due to ischemia, severe rejection or subclinical rejection predicted a mild degree of CAN at 1-year after transplantation. Beyond one year subclinical rejection became less common although still persisted in 12.3% of the biopsies at 10 years. Changes attributed to chronic calcineurin nephrotoxicity increased progressively with time.[13]

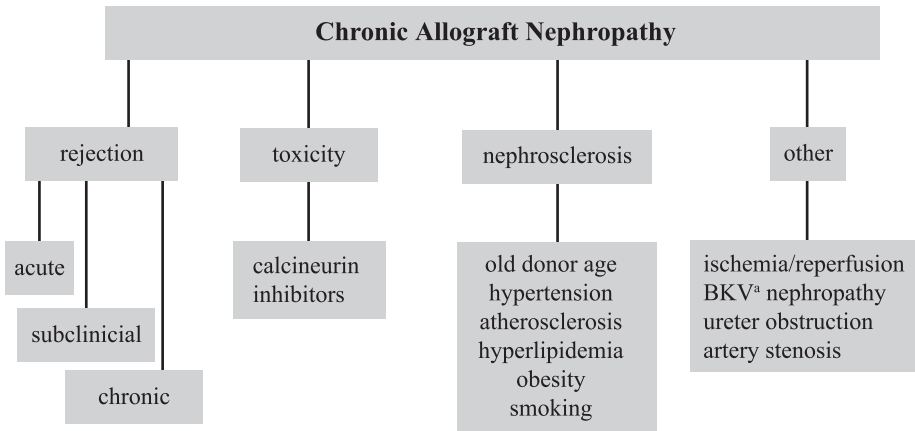

Figure 2: Factors that may induce CAN.

[a] BKV, polyomavirus type BK.

Table 1: Banff 1997. Chronic/sclerosing lesion scoring

Chronic Allograft Nephropathy (CAN)	
Grade	Histopathological Findings
Grade I (mild)	Mild interstitial fibrosis and tubular atrophy without (a) or with (b) specific vascular changes suggesting chronic rejection
Grade II (moderate)	Moderate interstitial fibrosis and tubular atrophy without (a) or with (b) specific vascular changes suggesting chronic rejection
Grade III (severe)	Severe interstitial fibrosis and tubular atrophy without (a) or with (b) specific vascular changes suggesting chronic rejection
Quantitative Criteria for Allograft Glomerulopathy ("cg")	
cg0	No glomerulopathy, double contours in <10% of peripheral capillary loops in most severely affected glomerulus
cg1	Double contours affecting up to 25% of peripheral capillary loops in the most affected of nonsclerotic glomeruli
cg2	Double contours affecting 26 to 50% of peripheral capillary loops in the most affected of nonsclerotic glomeruli
Quantitative Criteria for Interstitial Fibrosis ("ci")	
ci0	Interstitial fibrosis tissue in up to 5% of cortical area
ci1	Mild- Interstitial fibrosis tissue in 6 to 25% of cortical area
ci2	Moderate- interstitial fibrosis of 26 to 50% of cortical area
ci3	Severe interstitial fibrosis of >50% of cortical area
Quantitative Criteria for Tubular Atrophy ("ct")	
ct0	No tubular atrophy
ct1	Tubular atrophy in up to 25% of the area of cortical tubules
ct2	Tubular atrophy involving 26 to 50% of the area of cortical tubules
ct3	Tubular atrophy of >50% of cortical tubulus
Quantitative Criteria for Fibrous Intimal Thickening ("cv")	
cv0	No chronic vascular changes
cv1	Vascular narrowing of up to 25% lumenal area by fibrointimal thickening of arteries ± breach of internal elastic lamina or presence of foam cells or occasional mononuclear cells*
cv2	Increased severity of changes described above with 26 to 50% narrowing of vascular lumenal area*
cv3	Severe vascular changes with >50% narrowing of vascular lumenal area*
* in most severely affected vessel. Note if lesions characteristic of chronic rejection (elastica breaks, inflammatory cells in fibrosis, formation of neointima) are seen	

Quantitative Criteria for Mesangial Matrix Increase ("mm")*	
mm0	No mesangial matrix increase
mm1	Up to 25% of nonsclerotic glomeruli affected (at least moderate matrix increase)
mm2	26-50% of nonsclerotic glomeruli affected (at least moderate matrix increase)
mm3	>50% of nonsclerotic glomeruli affected (at least moderate matrix increase)
* The threshold criterion for the moderately increased "mm" is the expanded mesangial interspace between adjacent capillaries. If the width of the interspace exceeds two mesangial cells on the average in at least two glomerular lobules the "mm" is moderately increased	
Quantitative Criteria for Arteriolar Hyaline Thickening ("ah")	
ah0	No PAS-positive hyaline thickening
ah1	Mild-to-moderate PAS-positive hyaline thickening in at least one arteriole
ah2	Moderate-to-severe PAS-positive hyaline thickening in more than one arteriole
ah3	Severe PAS-positive hyaline thickening in many arterioles
Indicate arteriolitis (significance unknown) by an asterisk on ah	

A major challenge is to recognize the factors that are still operating in damaging the allografted kidney. The nature of the changes that are found in the biopsy in the vascular and glomerular compartments may sometimes be suggestive of the etiology of graft dysfunction. Chronic allograft glomerulopathy (CAG) indicates an alloantigen dependent insult at the level the glomeruli but is found in a minority (5-15%) of late biopsies with CAN.[14-18] At light microscopy it is characterized by glomerular enlargement, swelling of endothelial and mesangial cells, mesangiolysis, infiltration with mononuclear cells, mesangial matrix expansion and widening of the subendothelial zone with interposition of mesangial cells and matrix leading to characteristic basement membrane double contours.[19] Immufluorescence staining may show a non-specific pattern of IgM binding; in the vast majority staining for C4d (see below) is positive.[20-21] At electronmicroscopy (EM) an electron-lucent zone of fine floccular material in the glomerular subendothelial space is observed. Concentric intimal thickening of arteries and arterioles may result from chronic rejection (CR) but may also be donor-derived or result from cardiovascular risk factors present in the recipient.[22-23] The presence of inflammatory cells in a fibrotic intima, disruptions of the elastica, and myofibroblast proliferation resulting in the formation of a second neointima, are changes considered to be more specific for CR.[10] The evaluation of larger arteries is sensitive to sampling error. Nodular hyaline insudation in the periphery of small arterioles either patchy or circumferential designated as peripheral nodular hyaline

degeneration (PNHD) is a specific but probably not very sensitive sign of chronic CsA nephrotoxicity.[24-27]

During recent years, newer methods to define the cause of graft deterioration including immunostaining for C4d, a covalently bound fragment of a split product of the complement factor C4, and the EM examination of the peritubular capillaries (PTCs) have been under evaluation. The demonstration of C4d indicates local complement activation. Antibodies that bind to antigen trigger the assembly of C1qrs complexes that in turn catalyze the cleavage of complement components C4 and C2. C4b, generated in this way, forms amide or ester bonds with nearby proteins or saccharides, then associates with C2a to form the classical C3 convertase, C4b2a. Formation of C3 convertase on the surface of cells amplifies activation of complement but is also subject to various control mechanisms. Factor I together with a membrane bound co-factor protein, cleaves C4b to yield C4d, a catalytically inactive fragment. Complement-mediated injury of cells may also be prevented by changes in cellular metabolism induced by sublytic amounts of the membrane attack complex, which render cells less sensitive to complement-mediated injury.

It has recently been shown that the demonstration of the fragment C4d in the PTCs is a reliable tool for identifying a humoral component of acute rejection (AR).[28-32] It has been estimated that approximately 20 to 30% of all AR episodes have a humoral component, which adversely affects graft survival unless an intensified antirejection therapy with plasmapheresis (or immunoadsorption), mycophenolate mofetil, tacrolimus, or intravenous immunoglobulins is instituted. C4d staining has also been used to demonstrate a humoral contribution in chronic rejection. Positive C4d staining of the PTCs was found in 13, 34 or 61% in three retrospective studies of patients with presumed chronic rejection.[33-35] An association between the presence of C4d deposits in PTCs and CAG was reported in one of the studies,[35] which was not confirmed in another study.[36] Also an association with a high degree of multilayering of the basement membrane of PTCs has been reported (see below). At present, the significance of peritubular staining for the C4d in late biopsy samples with CAN is not fully established. The study of sequential biopsies of allografted kidneys has revealed that C4d deposits in the PTCs may appear or disappear at any time post transplantation.[36] The presence of capillary C4d in grafts biopsies taken late after transplantation had no prognostic significance, in contrast to the presence of C4d in biopsies taken within six months after transplantation.[37] Several authors have also emphasized that complement activation at the endothelial surface may not result in complement mediated damage.[38] It has been shown that allografts may accommodate to the presence of anti-donor antibodies.[39-43] C4d has been demonstrated in organ transplants with “accommodation”, *i.e.*, organs that function perfectly well despite the presence of circulating anti-donor blood group antibodies. The presence of C4d and the absence of immunoglobulin deposits or

other components of the complement system may also indicate that the metabolism of the endothelium has been modified to enhance the clearance of immune complexes. Additional studies of protocol biopsies need to be performed to demonstrate the importance of the C4d staining for the diagnosis of chronic humoral rejection in biopsies with CAN.

Recently it has been recognized that extensive reduplication of the peritubular capillary basement membranes (PTCR) is associated with CAG.[35,44,45] However, only a portion of patients with CAG shows well-developed PTCR.[46] In one study some biopsies with well developed PTCR did not display CAG,[46] which could not be confirmed in another study.[45] The significance of well-developed PTCR and its predictive value for CR in the absence of CAG remains to be established.

Polyomavirus type BK (BK virus) may reactivate from latency under immunosuppression and causes a chronic form of tubulointerstitial nephritis. BKV nephropathy occurs in a small percentage of patients, on average 6-18 months after transplantation and is related to intensified immunosuppression and multiple courses of antirejection therapy.[47] Patchy tubulointerstitial inflammation, characteristic intranuclear inclusion bodies, progressive tubular atrophy and interstitial fibrosis characterize the histology of BKN. Immunohistochemistry or EM can achieve diagnostic confirmation of BK-viruses infection. The presence of decoy cells in the urine and measurement of BKV DNA in the plasma are useful tools for early detection. In the same kidney BKN may coincide with tubulitis due to AR. The detection of transplant endarteritis is diagnostic of AR (Banff type II rejection). Interstitial mononuclear inflammatory cell infiltrates and typical tubulitis in areas lacking cytopathic changes are also suggestive of AR (morphological changes suggestive of Banff type I rejection). Additional histochemical studies to detect the tubular expression of MHC-class II (HLA-DR), can be used to establish a diagnosis of concurrent AR.[48] Tubules affected by AR show positive staining for HLA-DR antigens, whereas tubules affected by BKV do not. Currently, the prevalence of BKN in different transplant centers varies between less than 1 to 5.5%.[47]

Outline of the thesis

The studies as described in this thesis focussed on several issues: the contribution of chronic CsA nephrotoxicity to late allograft dysfunction and CAN; the pathogenesis of chronic CsA nephrotoxicity with special reference to direct tubulotoxicity; and possible differences in renal cortical interstitial matrix composition or cortical messenger RNA (mRNA) content of allografts that suffer from either chronic rejection or chronic CsA nephrotoxicity. The latter studies were done to explore if new tools could be developed in the differentiation of chronic rejection from chronic CsA nephrotoxicity.

In **chapter 2** an extensive and detailed literature review of chronic CsA nephrotoxicity after kidney allograft transplantation is presented.

In **chapter 3** the tubulotoxicity of CsA is studied in cultured human proximal tubular epithelial cells by assessment of cell death through either necrosis or apoptosis.

In **chapter 4** the impact of chronic CsA nephropathy on the incidence CAN and possible prevention by withdrawal of CsA after a critical time frame was studied. The chapter describes the results of the extended 15-year follow-up of an open-label, randomized trial that examined conversion to azathioprine as early as 3 months after transplantation.

Chapter 5 investigated whether the cortical ECM composition differs between allografts that lost function because of CR or chronic CsA nephrotoxicity. The cortical interstitial ECM composition of kidney allografts of three groups of patients was studied: those suffering from chronic CsA nephrotoxicity, those with chronic rejection and a third group of patients who were on cyclosporine medication but who were most likely to suffer from CR. The study investigated the proteins collagen I, III, and IV, collagen IVα3 and laminin β2 by immunohistochemistry with the use of a computerized morphometric method.

In **chapter 6** we investigated whether renal cortical mRNA levels of several proteins can serve as discriminating tools for chronic CsA nephrotoxicity or chronic allograft rejection. Total RNA was extracted from the cortex of renal biopsies, and mRNA levels of transforming growth factor β (TGF-β) and the extracellular matrix (ECM) molecules collagen Iα1, IIIα1, IVα3, decorin, fibronectin, and laminin β2 were measured by real-time PCR.

Chapter 7 summarizes and discusses the studies described in this thesis.

Chapter 8 gives a summary in Dutch.

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Chapter 2

Chronic Cyclosporine Nephrotoxicity in Renal Transplantation

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Abstract

Although extensively studied, the pathophysiologic characteristics of chronic cyclosporine A (CsA) nephrotoxicity are still far from being completely understood. The recognition of chronic CsA nephrotoxicity in allografted kidneys is hampered by a lack of easily assessable sensitive and specific markers. Long-term results of CsA withdrawal trials and trials that evaluated CsA sparing or withdrawal after the diagnosis of chronic allograft nephropathy (CAN) have shown that chronic CsA nephrotoxicity has a more important role in the etiology of late transplant dysfunction than appreciated before. Various hypotheses have explained the renal structural changes of chronic CsA nephrotoxicity including ischemia, cellular toxicity, and the stimulation of renal fibrosis by growth factors or cytokines. Possible ways to prevent chronic CsA nephrotoxicity include improved therapeutic drug monitoring and CsA withdrawal or avoidance. Patients with aspecific CAN in late biopsy may benefit from withdrawal of CsA or a reduction of its dose. Current knowledge is being discussed. It is concluded that in the near future more strategies are likely to be used to prevent loss of allograft function as a result of drug toxicity.

Introduction

Twenty-five years after its introduction in organ transplantation, cyclosporine A (CsA) is still one of the most widely used immunosuppressive drugs. The use of CsA-based immunosuppressive therapy has allowed significant improvement in the success rate of kidney transplantation, providing approximately 90% allograft survival at 1 year.[1] A major drawback of the drug, however, is its renal toxicity. Acute functional CsA nephrotoxicity is characterized by renal vasoconstriction and is largely reversible on dose reduction.[2] An irreversible decline in kidney function associated with irreversible pathologic changes may also occur after long-term CsA therapy.[3] In this review, we focus on the impact of chronic CsA nephrotoxicity on long-term allograft survival after kidney transplantation, its recognition and pathogenesis, and the current knowledge on strategies to avoid or ameliorate chronic CsA nephrotoxicity.

Impact of chronic CsA nephrotoxicity on long-term allograft survival

Late graft loss remains a major problem after kidney transplantation. About 50-60% of allograft loss after the first year of transplantation is explained by the death of the recipient, mainly resulting from a cardiovascular event. The second most common cause of late graft attrition is a transplantation-related condition designated as chronic transplant dysfunction (CTD), which accounts for 30 to 40% of late losses.[4] The condition is clinically characterized by a relatively slow but variable rate of decline in glomerular tranplantation rate (GFR), increasing proteinuria, and aggravated or new-onset hypertension. The cause may be multifactorial, and both alloantigen-dependent and alloantigen-independent mechanisms may be involved, including chronic CsA nephrotoxicity.

The specific understanding of the importance of chronic CsA nephrotoxicity in renal transplantation has long been hampered by the lack of specific and sensitive markers of this condition and the absence of studies with long-term follow-up. Chronic CsA nephrotoxicity may affect the allografted kidney rather slowly, and it may take many years before the real impact of chronic CsA nephrotoxicity is evident in clinical trials. However, several lines of new evidence suggest that chronic CsA nephrotoxicity may have a more prominent role in CTD than appreciated before.

It is now understood that acute rejection (AR) may have a detrimental effect on long-term kidney allograft survival, especially when it is multiple, is accompanied by vascular involvement, is late (after 3 months), or occurs in a kidney of a donor who is more than 50 years old.[5-6] The introduction of CsA (Sandimmune; Sandoz, Basel, Switzerland) significantly decreased AR rates, but long-term graft survival improved only modestly, suggesting an important nephrotoxic effect of the drug. After the subsequent use of the microemulsion formulation of CsA (Neoral; Novartis AG, Basel, Switzerland), drug delivery improved and an additional decrease in the number of AR episodes was observed. Large data base information found a better long-term graft survival since the introduction of Neoral.[7] However, a single-center conversion study that used antibody induction therapy found no difference in patient or graft survival, renal function or progression to chronic allograft nephropathy (CAN) at 5 years, probably because of an increase in nephrotoxicity.[8] In our own Sandimmune-Neoral conversion study, 20% of stable renal transplant patients treated with once-a-day low-dose CsA experienced chronic CsA nephrotoxicity after conversion to a twice-a-day Neoral regimen given according to the manufacturers guidelines.[9]

Data derived from studies on patients with various autoimmune diseases or solid organ transplants other than a kidney who were treated with CsA also indicate a relatively high incidence of chronic CsA nephrotoxicity.[10-14] Chronic renal failure (GFR

<29 mL/min·1.73 m^2) affected 7% to 21% within 5 years after transplantation of a nonrenal organ in the United States. In heart transplant patients, end-stage renal failure has been observed in 6-10%, the frequency of which apparently increases with a longer period of follow-up.[15] Heart transplant patients receive relatively high doses of CsA because of fear of rejection. However, renal functional or structural changes have also been observed frequently in patients on a regimen of lower doses of CsA for autoimmune disease. Two studies examined patients with psoriasis and included pretreatment and posttreatment protocol biopsies.[11,14] Biopsies taken at 1 year showed de novo interstitial fibrosis in more than 40% of patients in the study of Svarstad.[11] Zachariae *et al.*[14] reported a histologic follow-up of 25 patients. Seventeen patients had normal baseline histologic features, and at 2 years all had histologic changes compatible with chronic CsA nephrotoxicity. At 4 years, all studied biopsies (n = 11) displayed moderate to severe fibrosis. It should borne in mind that patients allografted with a single kidney may be even more susceptible to chronic CsA nephrotoxicity simply because they have a much smaller renal mass.

More direct evidence of the importance of chronic CsA nephrotoxicity as an etiologic factor of CTD has come from recently analyzed data in 2 azathioprine conversion studies with more than 10 years of follow-up that show a significant higher incidence of CAN and graft loss in the patients who continued on a regimen of CsA (discussed later),[16-17] and 2 studies that evaluated the conversion to mycophenolate mofetil (MMF) in patients with established aspecific CAN (also discussed later).[18-19] A recently published large study of protocol biopsies of patients who received a kidney-pancreas transplant identified 2 distinctive phases of tissue injury leading to CAN. An initial-phase early posttransplantation period with tubulointerstitial damage caused by ischemia, severe rejection, or subclinical rejection predicted a mild degree of CAN at 1 year after transplantation. Beyond one year, subclinical rejection became less common, although it still persisted in 12.3% of the biopsies at 10 years. Changes attributed to chronic calcineurin nephrotoxicity increased progressively with time.[20]

Pathology and diagnosis of chronic CsA nephrotoxicity

The structural lesions due CsA nephrotoxicity that are most frequently found in native kidneys are nonspecific and include tubular atrophy, interstitial fibrosis, slight mononuclear cell infiltration, Bowman's capsule basement membrane thickening, glomerular collapse, global sclerosis and nonspecific arteriolar hyalinosis as seen in hypertension or diabetes. Glomerular thrombosis or necrosis and signs of arteriolar thrombotic microangiopathy are rare with the use of lower doses of CsA.[21] Also, tubular alterations including honeycomb vacuolization

of the proximal tubular epithelium, giant mitochondria, and microcalcifications or minor changes of endothelial or smooth muscle cells now occur infrequently.[21] Reduplication and tangling of the arteriolar endothelial basal lamina on electron microscopy (EM) were reported in 1 study.[22] The most specific marker of CsA nephrotoxicity is nodular hyaline insudation in the periphery of small arterioles, either patchy or circumferential, designated as peripheral nodular hyaline degeneration (PNHD).[21] Arterioles in up to 2 layers of smooth muscle cells are involved and may become completely obstructed. EM has suggested that the deposits of PNHD occur at sites of myocyte necrosis.[21] Immunofluorescence staining is often positive for immunoglobulin M and C3,[21] representing nonspecific binding of plasma proteins. PNHD must be differentiated from the arteriolar hyaline changes as seen in diabetes or long-standing hypertension, in which the hyaline insudation occurs on the inside of the smooth muscle cell (SMC) layer.[21] The differentiation may be difficult on light microscopy, especially when the lesions are more pronounced, and EM may be needed. PNHD may regress or even completely vanish on reduction of dose or withdrawal of CsA.[23]

The hallmark of irreversible chronic CsA nephrotoxicity is the occurrence of tubulointerstitial and glomerular changes, including segmental and global glomerulosclerosis, interstitial fibrosis, and tubular atrophy.[21] The tubulointerstitial changes may be found before renal function is impaired.[10] In a rat model of chronic CsA nephrotoxicity, it was observed that these changes occur even before PNHD is noted.[24] The severity of the tubulointerstitial changes correlates with the degree of glomerular sclerosis.[22] Nonaffected glomeruli are hypertrophied[25] and presumably preserve function by hyperfiltration.

In a series of 192 patients treated with CsA for various autoimmune diseases, 26% of the biopsies showed interstitial fibrosis; however, PNHD was only noted in 4%.[26] Nonspecific arteriolar hyalinosis was observed more frequently. In another study with protocol biopsies of patients with uveitis, a high incidence of arteriolar hyaline changes was found that increased steadily with time.[14] However, in this study, it was not clear whether the specific PNHD lesion was scored. In 1994, an international advisory board of nephropathologists with extensive experience in the evaluation of kidney biopsies of patients on a regimen of CsA found that the reproducibility and diagnostic reliability of the evaluation of arteriolar lesions including PNHD were poor;[26] the interobserver variation on tubulointerstitial changes was low.

The diagnosis of chronic CsA nephrotoxicity in allografted kidneys is difficult because of the more frequently observed chronic rejection or nonspecific findings. Various insults that are alloantigen-dependent or alloantigen-independent may be operative at the same time, and it may be difficult to estimate the specific contribution of each of these factors. The nature of changes in the vascular and glomerular compartments may sometimes be suggestive of

an etiologic factor in graft deterioration. Allograft glomerulopathy with reduplication of the glomerular basement membrane indicates an alloantigen-dependent insult at the level the glomeruli but is found in a minority of late biopsies.[27] Concentric intimal thickening of arteries and arterioles may result from chronic rejection.[28] When PNHD is found, CsA nephrotoxicity should be considered. Striped fibrosis in renal allografts is not a marker of CsA nephrotoxicity because it has also been shown in a high proportion of late kidney allograft biopsies from patients maintained on a regimen of azathioprine and prednisone.[29] Recently, we studied the composition of the interstitial matrix of allografted kidneys with chronic CsA nephrotoxicity and chronic rejection. During chronic CsA nephrotoxicity, tubulointerstitial collagens III and IV accumulated preferentially, and no increase in collagen I was noted. An early increase in deposition of collagen I along with collagens III and IV was more specific for chronic rejection.[30]

Pathogenesis of chronic CsA nephrotoxicity

Several hypotheses have been proposed to explain the pathogenesis of the chronic CsA nephrotoxicity. These hypotheses are not mutually exclusive. Many supporting data are derived from rat models in which CsA nephrotoxicity has been extensively studied. Although the lesions of chronic CsA nephrotoxicity in rats resemble the lesions as seen in man,[31] there are important differences. Rats need much higher doses of CsA than humans, combined with salt depletion, or a special strain of spontaneously hypertensive rats has to be used. Therefore one should be cautious to extrapolate the data. Table 1 summarizes some of the work done in animals and humans.

The vascular hypothesis

This hypothesis assumes that chronic CsA nephrotoxicity is the result of ischemia. It is supported by morphologic and functional studies that report renal vasoconstriction, increased vascular resistance, decreased renal plasma flow, and pathologic alterations of renal arterioles. The fact that affected glomeruli appear shrunken, and that the fibrosis as studied in rats is initially patchy and occurs perpendicular from the corticomedullary junction may indicate a primary vascular etiology.[25] Additional evidence is derived from animal studies that show modulation of CsA-induced renal fibrosis by drug-induced modulation of intrarenal nitric oxide (NO) production (Table 1). Inhibition of NO production increased vasoconstriction and augmented fibrosis, whereas stimulation of NO production improved

Table 1: Experimental work on pathogenesis of CsA nephrotoxicity

Mediator, Enzyme, System*	**Experimental setting**	**Effect induced by CsA ***
RAAS	Rat kidney	JGA hyperplasia; increase renin content; increased AT_1 receptor reversed by antagonist; controversial amelioration of vasoconstrictor response by AT_1 antagonist or ACE inhibitor; less fibrosis with ACE inhibitor or AT_1 antagonist independent of blood pressure reduction; TGF-β1 expression decreased by AT_1 antagonist
	Rat plasma	Renin increased
	Human kidney	JGA hyperplasia, decreased renin after CsA withdrawal; AT_1 receptor antagonist; ACE inhibitor- no effect on vasoconstrictor response
	Human plasma	Renin increase controversial
	Human renal cortex fibroblasts culture	ACE inhibitor- reversed collagen synthesis
	Human PTEC culture	ACE inhibitor- reversed stimulated TGF-β secretion
TGF-β	Cultured cells, human and animal	Increased secretion
	In vivo expression, rodents and humans	Increased expression
	Gene polymorphism, humans	Affected degree of fibrosis
	Blocking antibody in rats	Fibrosis not reduced
Osteopontin	Rat model, CsA nephrotoxicity	Increase paralleling macrophage infiltration and fibrosis
	Human biopsy	No correlation with macrophage infiltration
MCP-1	Human allograft biopsy	Increased tubular expression
IGF-1	Cultured human renal fibroblasts	Production stimulated
	Receptor antibody, cultured fibroblasts	Collagen synthesis abrogated
IGF-1BP2 or 3	Cultured human renal fibroblasts	Secretion inhibited
PDGF	Cultured human tubular cells	Increased secretion
TIMP-1	Cultured human skin fibroblasts	mRNA up-regulated
	Biopsy rat model, CsA nephrotoxicity	Increased expression
PAI-1	Biopsy rat model, CsA nephrotoxicity	Increased expression in area of tubular atrophy
MMP-2 or 9	Cultured human renal fibroblasts	Reduced secretion
P-glycoprotein	Rat model, CsA nephrotoxicity	CsA induced P-glycoprotein expression; inverse correlation with severity of fibrosis and angiotensin II expression

Mediator, Enzyme, System*	Experimental setting	Effect induced by CsA *
NO	Endothelium-dependent vasodilatation *in vivo*, *in vitro*, *ex vivo* in humans and animals	Impaired but not in all studies
	Tissue, plasma, urinary metabolite levels in humans or animal models	Contradictory results
	Tissue NOS isoforms expression	iNOS decrease, eNOS increase *in vitro*, not in an animal model
	NO modulation, rat model, CsA nephrotoxicity	NO inhibition- more fibrosis, tubular apoptosis; NO enhancement- less fibrosis and tubular apoptosis, TGF-β_1 and PAI-1 downregulated
	L-arginine or L-NAME administration, animals	Improvement or worsening of renal hemodynamics
	L-arginine administration, human organ transplantation	No improvement of renal function or hemodynamics in 3 studies; 1 study showed improvement
Endothelin-1	Cultured renal cells	Increased secretion
	Human renal biopsy	Increased expression
	Urinary and plasma levels, rat and human	Elevated
	Receptors, rat kidney	Increased
	Endothelin 1 antibody treatment, rat	Partial relief of vasoconstriction
	Receptor blockade, rat	Partial relief of vasoconstrictor response, not all studies
	Receptor blockade, human	Modest increase in renal blood flow; no increase in GFR
	Blockade receptors in rat model	No effect on fibrosis
VGEF	Rat model, CsA nephrotoxicity	Increased expression in biopsy; VGEF administration ameliorated CsA-induced pathologic changes
ROS	Vit E administration in rat model	Reduced fibrosis and mRNA of TGFβ and osteopontin
Uric acid	Rat model, CsA nephrotoxicity	Pharmacologically induced hyperuricemia augmented fibrosis and arteriolar hyalinosis, increased renin, and decreased NOS-1 and 3 in rat kidney
Thromboxane A2	Blockade receptor in rat model, CsA nephrotoxicity	Less fibrosis

Table reference: See also reference 15 for addiotional references.

* Abbreviations: RAAS renin-angiotensin-aldosterone system,TGF-β transforming growth factor beta, MCP-1 monocyte chemoattractant protein 1, IGF-1 insulin growth factor 1, IGF-1BP insulin growth factor binding protein, TIMP-1 tissue inhibitor of matrix metaloproteinase 1, PAI-1 plasminogen activator inhibitor 1, MMP matrix metaloproteinase, P-GP P-glycoprotein, NO nitric oxide, VEGF vascular endothelial growth factor, ROS reactive oxygen species, JGA juxta glomerular apparatus, iNOS inducible nitric oxide synthetase, eNOS endothelial nitric oxide synthetase, NOS nitric oxide synthetase

renal blood flow and decreased fibrosis.[32] In our own Sandimmune-Neoral conversion study, it was found that the use of calcium channel and beta blockers reduced the risk of nephrotoxicity, independent from their effect on blood pressure. Both class of drugs may counteract CsA-induced vasoconstriction, which may be partly mediated by nervus truncus sympathicus activation.[9,3]

Direct cytotoxicity

CsA may directly damage renal cells and induce cell death and subsequent fibrosis. CsA is a highly lipophilic substance, which binds extensively to the cell membrane and its organelles and is concentrated in renal tissue 5- to -10-fold.[34] It may influence the physical properties of cellular membranes, but it does not seem to disrupt its integrity.[35] Ultrastructural morphologic studies of native kidneys have shown CsA-induced pathologic changes in endothelial cells, proximal tubular cells and smooth muscle cells. To study the direct cytotoxic effects, the drug has been added to cell culture systems. Usually, a broad concentration range was tested because of uncertainty of the concentration *in vitro* that corresponds to tissue concentrations *in vivo*. In the reported studies, differences in experimental conditions varied and sometimes major differences were evident, which could have been of relevance.

Endothelial cells

Several cell culture studies did not find a direct cytotoxic effect of CsA on human umbilical vascular endothelial cells at CsA concentrations up to 10 µg/mL.[36-37] In human umbilical vascular endothelial cells treated with a higher CsA concentration (12 µg/mL), an up-regulation of adhesion molecules (intercellular adhesion molecule 1 [ICAM-1], vascular cell adhesion molecule 1 [VCAM-1], and E-selectin) was observed together with increased adherence of leukocytes.[38] In a study of cultured rat endothelial cells, no toxicity was observed during a 6-day exposure period at maximum CsA concentrations of 1 µg/mL.[39] However, other studies did report cytotoxicity in bovine aorta endothelial cells at 1.2 µg/mL,[40] or 12 µg/mL.[41] Cell death of bovine glomerular endothelial cells was also reported, occurring at 1.2 µg/mL and within 3 hours[42] and suggested that endothelial cells derived from glomeruli are more sensitive. Further evidence for endothelial cytotoxicity is derived from studies that show an increase of various plasma markers of endothelial dysfunction that tend to normalize after CsA withdrawal.[43] Another study reported an

elevated number of circulating endothelial cells compared with levels in allografted patients not receiving calcineurin inhibitor therapy or in normal control subjects.[44] Although these data are somewhat contradictory, it can still be suspected that CsA is cytotoxic for endothelial cells, especially at high concentrations.

Tubular cells

Morphologic alterations of proximal tubular epithelial cells (PTEC) and a higher rate of tubular apoptosis in biopsies with CsA nephrotoxicity[45-46] have suggested that CsA could be directly involved in tubulotoxicity. The lack of tubular atrophy in the absence of significant glomerular sclerosis in human studies, however, argues against a direct tubulotoxic effect.[22]

Contradictory results have been reported in cell culture studies that used PTEC from various nonhuman and human sources. Two human studies reported loss of viability at a CsA concentration of 0.05 or 1 μg/mL,[47-48] whereas another study found no toxicity, despite the fact that higher concentrations (up to 10 μg/mL) were used.[49] The variance may be explained by differences in experimental protocols. In the positive studies, PTEC were deprived of essential culture supplements before incubation with CsA[47] or were of fetal origin.[48] Recently, necrosis or apoptosis was studied in cultured adult human PTEC.[50] No direct toxic effect of CsA was shown at concentrations up to 10 μg/mL, whereas higher concentrations proved to be toxic because of the vehicle Cremophore EL.

Smooth muscle cells

CsA stimulates the contraction and proliferation of cultured rat smooth muscle cells through an endothelin 1-dependent pathway and likewise the proliferation of human pulmonary artery smooth muscle cells, without significant toxicity at concentrations up to 0.12 μg/mL.[51-53] Two studies reported cytotoxicity at slightly higher concentrations up to1 μg/mL.[51-54] In 1 of these studies, no proliferative effect of CsA on smooth muscle cell growth was found. [54] Another study reported visceral smooth muscle cell dedifferentiation depending on calcineurin inhibition.[55] The overall data on smooth muscle cells are limited and lack a proof of cytotoxicity.

Growth Factors and Cytokines

Cyclosporine A may induce tissue remodeling by growth factor or cytokine release (Table 1). Results from *in vivo* and *in vitro* studies have suggested that CsA may stimulate matrix deposition independently of morphologic or functional vascular changes. In rats, CsA stimulates early interstitial matrix deposition, which precedes PNHD development and occurs independently of the hemodynamic changes.[24,56,57] It has been shown that angiotensin II plays a prominent role in chronic CsA nephrotoxicity in rodent models of chronic CsA nephrotoxicity.[57-60] Coadministration of angiotensin-converting enzyme (ACE) inhibitors or angiotensin II type 1 receptor antagonists minimize tubulointerstitial fibrosis independently of renal hemodynamic changes.[58-60] *In vitro* experiments with renal resident cells derived from rodents, primates, and humans have demonstrated that CsA stimulates the production of collagen.[47,61] Also, evidence for an impairment of matrix degradation has been found in cell culture studies[47,62] and in a rat model of CsA nephrotoxicity.[63] Various growth factors have been implicated in this scarring process. CsA stimulates the expression of transforming growth factor β1 (TGF-β1) by renal resident cells and macrophages.[24,64,65] This increase in TGF-β1 expression may be driven by angiotensin II,[66] or by endothelin.

There is abundant evidence that CsA stimulates endothelin production.[67] Blockade of endothelin receptors in a rat model of CsA nefrotoxicity, however, did not result in a decrease in fibrosis, which draws into question the importance of this endothelin-TGF-β1 interaction.[57,68] Losartan or enalapril decreased TGF-β1 messenger RNA (mRNA) and decreased extracellular matrix deposition in a rat model,[59] emphasizing a central role of angiotensin II in rodents. Pirfenidone, a novel antifibrotic compound, decreased TGF-β1 mRNA and protein expression and ameliorated fibrosis in CsA-treated rats.[69] However, the use of TGF-β1 antibodies did not change the extent of tubulointerstitial fibrosis despite a decrease in PNHD and preservation of function.[64] In cultured human PTEC, enalapril prevented the CsA-induced TGF-β 1 secretion.[70] Likewise, TGF-β 1 plasma levels decreased in renal allograft recipients with losartan treatment.[71] Angiotensin II also stimulates (CTGF) gene expression. However, this pathway is shown to be calcineurin-dependent and directly inhibited by CsA itself, which makes it a less likely candidate to be involved in chronic CsA nephrotoxicity.

CsA treatment of cultured human renal cortical fibroblasts stimulated insulin-like growth factor 1 (IGF-1) secretion and inhibits secretion of IGF-1 binding proteins 2 and 3.[47] CsA significantly stimulated collagen synthesis in the same model and inhibited the expressions of enzymes involved in matrix degradation. CsA did not affect TGF-β1 protein secretion in the same study. An anti-IGF-I receptor antibody abrogated increased collagen synthesis. In human PTEC, CsA also stimulated the secretion of the fibrogenic cytokine platelet-derived growth factor.[47]

Significant infiltration with mononuclear cells occurs before the development of interstitial fibrosis in rats with chronic CsA nephrotoxicity.[72] The macrophage chemoattractant osteopontin was found to be upregulated in PTEC and correlated with the degree of macrophage infiltration and fibrosis development in rats.[58] However, in humans no correlation was found between tubular osteopontin expression and monocyte-macrophage infiltration.[73] In biopsies of human kidney transplant recipients who were believed to have chronic CsA toxicity, intense tubular staining for endothelin 1, RANTES (regulated upon activation, normal T-cell expressed and secreted), and monocyte chemoattractant protein 1 mRNA was found in areas of tubular atrophy and fibrosis. So far, it is not clear what triggered the upregulation of these molecules.[74]

Prevention of Chronic CsA Nephrotoxicity

Currently, calcineurin inhibition is still a cornerstone in immunosuppressive therapy after kidney transplantation. Possible ways to prevent chronic CsA nephrotoxicity include a better control of CsA exposure, withdrawal of CsA after a critical time frame, or the complete avoidance of CsA. Also, the use of tacrolimus instead of CsA may be considered.

Improved therapeutic drug monitoring

Almost 25 years of CsA therapy has been monitored in trough blood levels (C0). C0 levels were kept within the "therapeutic range", with differences in target levels in various transplantation centers (in the United States, often 150-250 μg/L; in Europe, 100-200 μg/L), but a significant number of patients experienced a lack of efficacy or renal toxicity. Data from the Collaborative Transplant Study have indicated that the 1-year CsA dose is significantly associated with long-term graft survival with evidence of a worse prognosis at doses less than 3 mg/kg per day or higher than 6 mg/kg per day.[75] Recently, it has been shown that C0 levels correlate poorly with systemic CsA exposure as measured by the area under the 12-hour concentration versus time curve [(AUC(0-12)] because of extensive interpatient and intrapatient variability in CsA absorption and metabolism.[76] A number of patients are overexposed or underexposed to the drug when C0 monitoring is used. These data suggest that there could be a therapeutic window for individual dosing that combines maximum efficacy with minimal toxicity. Neoral, the microemulsion preparation of CsA, displays a predictable absorption profile with the absorption phase of the drug having the greatest interpatient and intrapatient variability. The 4-hour AUC [AUC(0-4)]

as estimated by limited sampling models (LSMs) has been shown to correlate closely with systemic exposure.[77] Inadequate CsA exposure is a major risk factor for (subclinical) AR, which predisposes to late allograft failure.[78] Neoral dosing based on AUC(0-4) measurement conveys a higher efficacy and lower risk of toxicity of the drug early after transplantation than dosing on C0 levels.[79] The concentration 2 hours after ingestion (C2 level) is a good predictor of the absorption phase measured by the AUC(0-4), and C2-guided dose adjustments resulted in much lower AR rates early post-transplantation.[80-81] Newer studies show that achieving AUC(0-4) values of 4400 to 5500 μg·h/L or C2 levels of 1500 to 2000 μg/L during the first 3 days after transplantation minimizes the risk of rejection and improves graft function.[80,82,83] However, in comparison with C0 levels, the single-point C2 level does not correlate better with total systemic exposure to CsA as measured by AUC(0-12).[84] C2 monitoring thus prevents underexposure and provides higher efficacy but does not give better protection than C0 monitoring against overexposure of the drug with the risk of long-term nephrotoxicity. As compared with C0 monitoring, the use of LSMs has improved the estimation of systemic exposure, but equations are rigid and not reliable in patients with an abnormal absorption profile. A compartmental population pharmacokinetic model for CsA in renal transplant recipients combined with the maximum a posteriori Bayesian fitting method seems more practical because it offers the important advantage of flexibility in sampling times after drug administration and provides the opportunity for long-term AUC-guided dosing.[84] The performance of this model is comparable to that of LSMs in kidney transplant patients and superior in SPK recipients. Measuring CsA concentrations at the time points 0, 2 and 3 postdose hours provides an excellent estimation of the AUC(0-12). However, it remains to be shown that therapeutic drug monitoring of CsA based on AUC estimation will provide protection against long-term CsA nephrotoxicity.

Withdrawal studies

An alternative approach is to reduce the exposure to CsA after the period of the highest chance of AR. A number of clinical trials have examined the safety of CsA withdrawal from dual therapy with steroids and replacement by azathioprine. Improvements in renal function, lipid profile, hypertension, and the incidence of gout were reported.[85] A 10% increase in the AR rates was also observed. However, a higher rate of subsequent graft loss was not reported.[85] Recently analyzed 15-year data from a single-center, open-label, prospective randomized study that compared CsA continuation with conversion to azathioprine 3 months after transplantation showed a higher risk of CAN in the group that continued

CsA (relative risk, 4.3; 95% confidence interval, 1.4-12.9).[16] In the study, predominantly white recipients were included who were generally well matched for human leukocyte antigens. A better death-censored graft survival was observed in the azathioprine group after 2 post-transplantation years. The 15-year death-censored graft survival was 81.9% *vs* 69.2% ($P = 0.012$). The study showed that long-term allograft function is better preserved after conversion to a calcineurin inhibitor-free immunosuppressive regimen. These results are in line with data from a not yet published multicenter Australian conversion study with an identical follow-up. In this study, a highly significant difference in mean graft survival was found favoring CsA usage shorter than 6 months (13.3 *vs.* 11.8 years, $P < 0.01$).[17]
CsA withdrawal from a triple regimen has also been studied and resulted in a similar increase in AR rates.[86] It was found that MMF continuation instead of a switch to azathioprine provided better protection against AR.[87] Another drug that has been used after the withdrawal of CsA is sirolimus. The use of sirolimus in a triple-drug regimen together with CsA and steroids directly after transplantation results in a lower incidence of AR than when azathioprine is used.[88] However, a mild adverse effect on renal function has been noted, which is in line with earlier animal studies that showed increased CsA nephrotoxicity.[89] This adverse effect may be explained by a pharmacokinetic interaction of sirolimus with CsA that increases systemic CsA exposure and CsA at the tissue level. Both drugs are substrates for P-glycoproteins and for cytochrome P-450 3A4 and are mutually competitive, mostly at the gut level, increasing each other's oral bioavailability.[90] The administration of either drug at 4-hour intervals may minimize the interaction in the absorption phase, but at the tissue level a second interaction occurs. Sirolimus increases renal tissue concentrations of CsA, whereas CsA decreases the sirolimus concentration.[91] The specific interaction of sirolimus with CsA at the tissue level impairs precise pharmacodynamic titration of CsA by whole-blood levels. Results for CsA withdrawal at 3 months from a sirolimus-containing triple regimen have been reported.[92] A non-significant 6% increase of AR was noted, but at 1 year a better GFR (difference, 6 mL/min) was seen in the group that was withdrawn from CsA. The 3-year results showed a 6% higher graft survival ($P = 0.052$) and a significantly higher GFR of 12 mL/min.[93] CsA therapy in this study was monitored on C0 measurements, and the study did not include highly sensitized patients or patients with severe rejection 4 weeks previously; also, there were few black recipients, no HLA-identical patients, and no patients with poor allograft function.

Tacrolimus-Based Vs CsA-based Immunosuppression

Several studies reported a lower incidence and lesser severity of AR early after transplantation with a tacrolimus-based immunosuppressive regimen as compared with CsA-based treatment irrespective of the type of CsA formulation used.[94] A higher GFR and a reduced requirement for antihypertensive and lipid-controlling medication were also recorded. A better graft survival at 1 year after transplantation was found in pediatric patients receiving tacrolimus compared with those receiving the CsA microemulsion formulation.[95] The 3-year kidney allograft survival in adult patients receiving tacrolimus was higher only in the patients who experienced delayed graft function.[96] Another study reported equivalent graft survival at 5 years, but a higher incidence of treatment failure was observed during CsA therapy, which led to higher crossover to tacrolimus.[97] Yet another study found no difference in graft survival at 5 years in pairs of kidneys allocated to either initial tacrolimus or initial CsA treatment; however, treatment failures were not examined.[98] In protocol biopsies, a higher degree of allograft fibrosis was reported at 1 year after transplantation in patients on a regimen of CsA compared with those on a regimen of tacrolimus,[99] whereas in a different study of 2-year biopsies, no difference in CAN score or subclinical rejection was observed.[100] These data are still inconclusive on the long-term benefit of tacrolimus-based therapy over CsA-based therapy. Early posttransplantation tacrolimus seems to provide a higher level of protection against rejection; however, newer ways of therapeutic drug monitoring were not used in any of the mentioned studies.
An important trade-off of tacrolimus-based therapy is the higher incidence of posttransplant diabetes mellitus, which may have an adverse effect on graft and patient survival.[101] Tacrolimus may also induce chronic calcineurin inhibitor nephrotoxicity with pathologic characteristics similar to those of chronic CsA nephrotoxicity.

Avoidance of Calcineurin Inhibitors

Long-term CsA nephrotoxicity could be prevented by complete avoidance of the drug. A prerequisite is the use of an alternative equipotent immunosuppressive drug with an acceptable safety profile. A recent study on sirolimus-based therapy suggested that the drug has antirejection efficacy similar to that of CsA and at 6 and 12 months the GFR was higher in the patients receiving sirolimus.[102] Long-term results of studies on calcineurin-free immunosuppressive therapy are still lacking.

CsA sparing in patients with established CAN

CsA sparing in patients with established CAN has been studied with MMF as the sparing agent.[18] The slope in GFR decline before and after intervention was compared. Fifty to sixty percent of the patients treated with either a reduced dose of CsA (n = 67) or tacrolimus (n = 33) showed an improvement in the rate of decline; more than 90% of the patients who stopped calcineurin inhibitor medication (n = 18) showed a slowing of the loss of function. A small risk of AR was noted. Another ongoing multicenter study examined the withdrawal of CsA in patients with chronic gradually declining allograft function. After 6 months, stabilization or improvement was seen more often (58% *vs* 32%) in the patients who were converted to MMF (n = 73) as compared with the ones that continued on CsA (n = 70). No significant differences in AR, graft loss, or death were observed.[19] These studies show that it may be advantageous to withdraw patients from CsA when allograft function is declining and renal biopsy indicates aspecific signs of CAN.

Conclusions

CsA-based immunosuppressive therapy is associated with significant long-term nephrotoxicity. The recognition of chronic CsA nephrotoxicity in allografted kidneys is still imperfect and needs to be improved. New strategies to prevent toxicity such as CsA withdrawal after a critical period, improved therapeutic drug monitoring, and the use of a calcineurin inhibitor-free immunosuppressive regimen are likely to be implemented more often in the near future. Patients with aspecific CAN in late biopsy samples may benefit from withdrawal of CsA or a reduction of its dose in conjunction with MMF administration.

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Chapter 3

Renal tubular epithelial cell death and Cyclosporine A

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Abstract

Background: The pathogenesis of chronic cyclosporine A (CsA) nephrotoxicity is largely unknown. In this study we examined whether CsA produces cell death through necrosis or apoptosis of either cultured human proximal tubular epithelial cells (PTEC) or the porcine tubular cell line LLC-PK_1.

Methods: Primary isolates of human PTEC and LLC-PK_1-cells were treated for various time periods with CsA at concentrations of 0.01-100 µg/ml. Apoptosis was studied by the assessment of annexin binding and propidium iodide uptake, the measurement of cellular DNA content and cell cycle analysis, and by the evaluation of nuclear morphology. Cell death was studied by the trypan blue exclusion method. Hypoxic conditions were simulated through chemical ATP depletion.

Results: In human PTEC, cell death was observed at CsA concentrations higher than 10 µg/ml; at these concentrations PTEC died as a result of necrosis and toxicity of its vehicle Cremophore EL, and not as a result of CsA inducing apoptosis. The addition of cycloheximide to relief a possible block in the apoptotic process had no effect on human PTEC, but did result in apoptosis of LLC-PK_1. In human PTEC, CsA did not augment cell death induced by chemical ATP depletion.

Conclusions: The results of this *in vitro* study do not support the hypothesis that CsA directly induces cell death of proximal tubular epithelial cells.

Introduction

Cyclosporine A (CsA) is one of the most widely used drugs in organ transplant patients.[1] CsA-based immunosuppressive regimens are associated with 1-year success rates for kidney transplants of ~ 90%,[2] but a major drawback is CsA renal toxicity. Acute CsA nephrotoxicity is characterized by renal vasoconstriction and is largely reversible upon dose reduction.[3] An irreversible decline in kidney function may also be observed after long-term CsA use and is associated with structural changes such as interstitial fibrosis, tubular atrophy, arteriolar hyalinosis and glomerulosclerosis.[4]

The exact pathogenesis of chronic CsA nephrotoxicity remains unknown.[1] Morphological studies reported proximal tubular epithelial cell vacuolization and inclusion bodies early after transplantation during CsA treatment, and animal and human studies have found

an increase in the urinary excretion of the proximal brush border enzyme *N*-acetyl-β-D-glucosaminidase.[5] Moreover, the urinary excretion of β_2-microglobulin is enhanced during CsA therapy, suggesting proximal tubular cell damage.[6] It has recently been hypothesized that a high concentration of CsA directly induces tubular cell necrosis and that a lower therapeutic concentration of the drug promotes apoptosis.[7] In both human and animal studies, a higher rate of tubular cell apoptosis has been described during CsA exposure;[8-9] however, it is still not clear whether this increased apoptotic activity is the result of a direct toxic effect of CsA or the result of an indirect mechanism such as ischemia. The aim of the present study was to examine whether CsA directly induces cell death of cultured proximal tubular epithelial cells by either necrosis or apoptosis.

Materials and methods

Materials

CsA was obtained as Sandimmune®, containing Cremophore EL and alcohol as vehicle (2:1) (Novartis Pharma B.V. Arnhem, The Netherlands), and as a powder (Sigma, St Louis, MO, USA) which was dissolved in ethanol. The mouse monoclonal antibody anti-Fas15 was a gift from Prof. L.A. Aarden (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Antimycin A (AA), 2-deoxy-D-glucose (DOG) and cycloheximide (CHX) were obtained from Sigma.

Cell cultures

All cell cultures were performed in an incubator using a humidified 5% CO_2/95% air mixture at 37°C. Human primary proximal tubular epithelial cells (PTEC) were obtained from pre-transplant renal biopsies as described previously.[10] In brief, small fragments of pre-transplant biopsies were placed in 25 cm^2 flasks (Costar, Cambridge, MA, USA) coated with a matrix of type I bovine collagen (Sigma) and decomplemented fetal calf serum (FCS; Gibco BRL, Breda, The Netherlands) in Dulbecco's modified Eagle's medium (DMEM/HAM-F12 at a ratio of 1:1 (Seromed, Biochrom KG, Berlin, Germany) supplemented with insulin (5 μg/ml), transferrin (5 μl/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), tri-iodothyronine (4 pg/ml) and epidermal growth factor (10 ng/ml) (all from Sigma). Medium was replaced every 3 days. The cells grown from the biopsied tissue showed

the characteristic morphology of tubular cells and immunofluorescence staining confirmed their proximal descent (Figure 1A and 1B). Subculturing of these cells was performed in the same type of medium using 25 and 75 cm^2 flasks (Costar) coated with FCS only. PTEC between passage 2 and 7 were used for the experiments. The porcine cell line LLC-PK_1 was kindly provided by Dr Michael P. Ryan (Department of Pharmacology, University College Dublin, Ireland). The cell line was originally obtained from the ATCC (Manassas, VA, USA), and cells have the characteristics of renal PTEC.[11] LLC-PK_1 cells were subcultured in 75 cm^2 flasks (Costar) using DMEM culture medium (Seromed) supplemented with 10% (v/v) decomplemented FCS. LLC-PK_1 cells were used between passage 210 and 230.

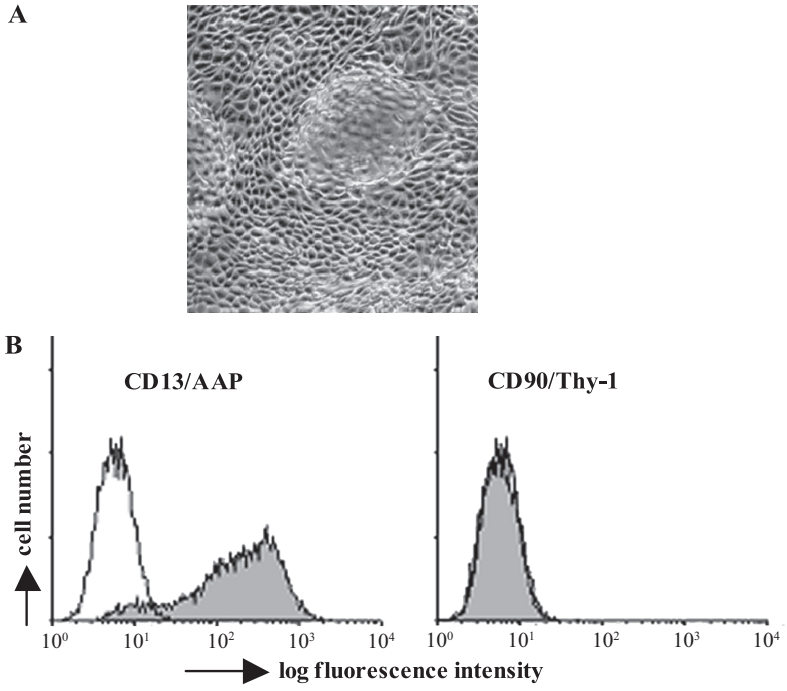


Figure 1: (**A**) Morphological appearance of primary cultures of human PTEC. The characteristic "dome" is the cell layer that has been lifted from the solid surface as a result of active ionic transport processes. **(B)** FACS analysis using a monoclonal antibody against alanine aminopeptidase (CD13), a cell surface marker that distinguishes proximal from distal TEC but not from fibroblasts, and an antibody against Thy-1/CD90 that is present on fibroblasts but not on TEC. The grey area under the curve represents cells that were incubated with the specific monoclonal antibody, while the white area represents cells that were incubated with the secondary antibody only (see Material and methods). The strong staining for CD13 and the absence of staining for CD90 confirms the proximal descent of the cultured TEC.

Fluorescence-activated cell sorter (FACS) analysis

For FACS analysis, cells were harvested by brief trypsinization to prevent proteolysis of surface receptors. After the cells were washed twice with FACS buffer (1% BSA, 1% decomplemented normal human serum, 0.02% sodium azide in PBS), 10^5 cells were incubated with specific monoclonal antibodies against either alanine aminopeptidase (CD13) or Thy-1/CD90 (AS02, Dianova-Hamburg, Germany). After incubation for 45 min at 4°C, cells were washed twice with FACS buffer and subsequently incubated with goat anti-mouse Ig-PE (DAKO) for 30 min at 4°C. Finally, the cells were washed, fixed with 1% paraformaldehyde, and assessed for fluorescence using a FACScan and LYSIS-II software (Becton Dickinson, Mountain View, CA, USA).

Cell treatments

For viability and apoptosis assays, cells were washed with PBS, trypsinized and seeded at a concentrations of 1.5 x 10^5 (human PTEC) or 0.5 x 10^5 (LLC-PK_1 cells) in 24-well plates (Greiner, Frickenhausen, Germany) coated with FCS, and grown for 24 h to assure culture subconfluence. They were then washed with PBS and treated for 24 h with CsA dissolved in culture medium, in a humidified incubator supplying a 5% CO_2/95% air mixture at 37°C. CsA-containing solutions were prepared by direct dilution of the clinical formulation Sandimmune® (CsA 50 mg/ml in Cremophore EL and ethanol 2:1)) in culture medium or by dissolution of CsA powder (Sigma) in absolute ethanol (5 mg/ml), with further dilutions made in culture medium. The final concentrations achieved were checked by a radioimmunoassay and the biological activity was measured in an OKT_3 T-cell proliferation assay. Inhibition of T cell proliferation was found with CsA dilutions up to 0.01 µg/ml.
To induce a state resembling tissue hypoxia *in vivo*, cultured cells were ATP depleted with the use of glucose-free culture medium and the addition of 2 µM Antimycine A, an inhibitor of the mitochondrial respiratory chain, and 5 mM 5-deoxy-D-glucose, an inhibitor of glycolysis.

Evaluation of cell viability

Cell viability was evaluated using the trypan blue exclusion assay. In brief, spontaneously detached cells and cells obtained after trypsinization were pooled and tested visually for their ability to exclude the dye. Cells that stained with trypan blue were considered dead.

Detection of apoptosis

After the culture supernatant was harvested, cells were washed in PBS and trypsinized to single cell suspensions. Trypsin was subsequently inactivated by the addition of culture medium supplemented with 10% FCS. PBS and the cell suspension were pooled with the supernatant and pelleted by centrifugation for 5 min at 230 *g*.

For morphological assessment, cells were fixed with 1% paraformaldehyde and kept on ice for at least 10 min. Cytospin specimens were prepared, stained for 3 min with Hoechst 33258 and evaluated by fluorescent microscopy.

For the assessment of phosphatidylserine externalization, cells were washed in 1 ml annexin buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4 adjusted at 4°C), resuspended in 50 µl FITC-annexin V (Nexins Research, Kattendijke, The Netherlands) (1/250 in binding buffer) and incubated for 15 minutes in the dark on ice. Prior to measurement, 100 µl of propidium iodide (PI; Molecular Probes, Leiden, The Netherlands) diluted in annexin buffer (final concentration 1 µg/ml) was added. Labeled cells were analyzed on a FACScan using the Lysis II software. The percentage of cells binding FITC-annexin V and/or PI was calculated using the WinMDI2.7 software. Cells that were negative or positive for both dyes were considered live or dead, respectively, while apoptotic cells were only positive for FITC-annexin V.[12]

To evaluate cellular DNA content, cells were washed and resuspended in 100 µl of 1 mM EDTA/PBS at 4°C, fixed by adding 700 µl 100% ethanol at –20°C and incubated for 30 min at –20 °C. Subsequently, cells were washed twice in 1 mM EDTA/PBS and resuspended in 300 µl PBS to which the following was added: EDTA (1 mM), PI (10 µg/ml) and RNAse A (50 µg/ml; Sigma). After a 45 minutes incubation at room temperature, cells were analyzed on a FACScan. The fraction of cells in each phase of the cell cycle was calculated according to cell DNA content using the WinMDI2.7 software.

Results

The effect of CsA on the viability and mode of cell death of primary isolates of human PTEC

Primary cultures of human PTEC were obtained from biopsies taken at kidney transplantation.[10] The proximal tubular descent of the cells growing out the tissue was confirmed by their characteristic epitheloid cell shape, their ability to form domes (Figure

1A) and their immunofluorescence staining for alanine aminopeptidase (CD13), a cell-specific marker for PTEC. Isolates were cultured for 24 h in the presence of increasing concentrations of the clinical formulation Sandimmune®, and tested for their ability to bind annexin V and to take up PI. As an apoptosis control we used the mouse monoclonal antibody anti-Fas15 (1 μg/ml) combined with cycloheximide (10 μg/ml) dissolved in culture medium, as described previously.[12]

At CsA concentrations of 10 μg/ml or lower, no significant increase in either annexin V binding or PI uptake was observed (Figure 2). Similar results were obtained when the number of cells seeded in the wells was reduced to 25%, the incubation period with CsA was extended to 72 h, or 20% (v/v) serum added to the media during CsA exposure (results not shown). A significant increase in the number of annexin V, PI positive cells (71% *vs* medium control 6.8%) was noted when a CsA concentration of 100 μg/ml was used. In the apoptosis control, significantly more cells stained for annexin V alone or for both dyes.

Subsequently, the DNA content of the cells was examined after 24 hours of incubation with CsA and the stage of the cell cycle was analyzed (Figure 3). A significant increase in the number of cells with a reduced DNA content (sub-G_0/G_1 fraction) was observed only at the highest CsA concentration (100 μg/ml). Next, we incubated PTEC for 24 h with increasing concentrations of CsA, and tested for dead cells by the use of the trypan blue exclusion method or for apoptosis by the evaluation of nuclear morphology (Figure 4). As expected, a concentration-dependent increase in cell death was observed at concentrations >10 μg/ml. However, no increase in the number of apoptotic cells was found (Figures 4 and 5).

The effect of CsA vs its vehicle on the viability of primary isolates of human PTEC

Next we compared the cytotoxicity of CsA or its vehicle Cremophore EL on human PTEC. PTEC were incubated for 24 h with high concentrations of Sandimmune, starting at 10 μg/ml, or its vehicle at comparable dilutions. Cell death was determined by the trypan blue exclusion assay (Figure 6). The vehicle itself exerted a profound cytotoxic effect, which was at least equal to the effect of Sandimmune at comparable dilutions. To examine the toxicity of CsA alone, CsA powder was dissolved in alcohol and diluted further in culture medium. PTEC were incubated with increasing concentrations of CsA for 24 h. Concentrations up to 10 μg/ml did not result in an increased rate of cell death, as assessed by the trypan blue method or in an increase of cells that displayed morphological signs of apoptosis (data not shown). A higher concentration could not be tested because of the inability to dissolve CsA. These results suggest that in primary isolates of human PTEC, the acute cellular toxicity of CsA at concentrations >10 μg/ml is mainly the result of vehicle toxicity and is not caused by the drug.

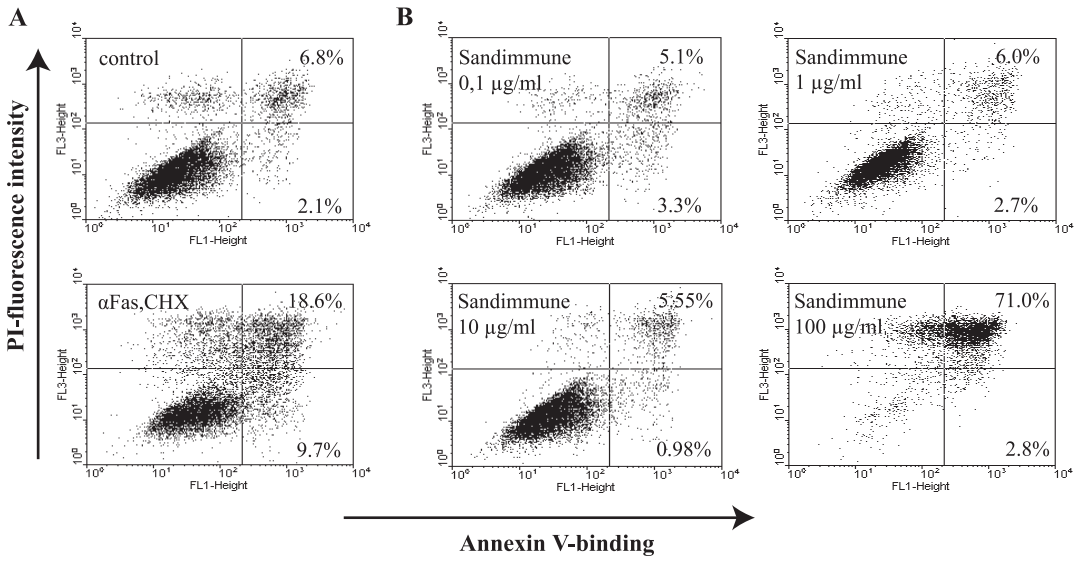


Figure 2: The effect of Sandimmune® on apoptosis or cell death of PTEC, as assessed by flow cytometric analysis of FITC-annexin V binding and PI staining. Cells were treated for 24 h with medium, the anti-Fas15 monoclonal antibody combined with cycloheximide 10 µg/ml (**A**) or increasing concentrations Sandimmune (**B**) Bottom-right quadrants: cells with externalized phosphatidylserine but still with an intact cell membrane, indicative of cells in early apoptosis. Top-right quadrants: cells positive for both dyes, *i.e.* late apoptotic or necrotic cells.

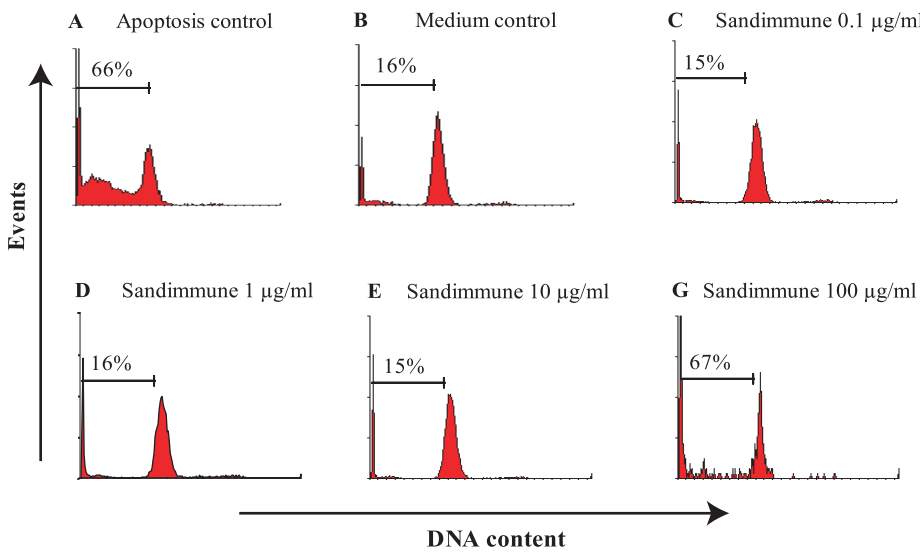


Figure 3: The effect of Sandimmune® on the cell cycle of human PTEC. Human PTEC were incubated for 24 h with the anti-Fas15 monoclonal antibody combined with cycloheximide (**A**) medium (**B**) or increasing concentrations of CsA (**C-F**) and the DNA contents of the cells was analyzed on a Facscan. An increase in the number of cells with reduced DNA content was observed at a Sandimmune concentration of 100 µg/ml and in the apoptosis control.

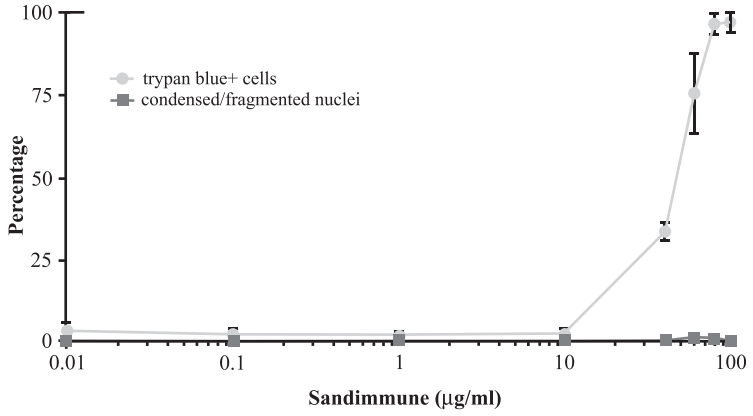


Figure 4: The effect of Sandimmune® on cellular viability and nuclear morphology of human PTEC. Cells were exposed to increasing concentrations of Sandimmune for 24 h. Cell death was measured by the trypan blue exclusion assay, and apoptosis by analysis of nuclear morphology using fluorescence microscopy after staining with Hoechst 33258. Results are expressed as the mean ± SEM of a representative experiment, performed in triplicate wells (n = 3).

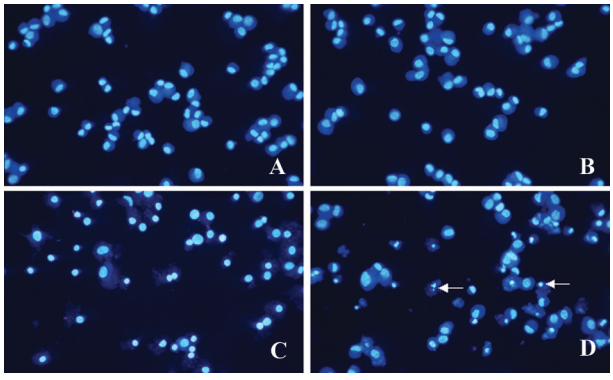


Figure 5: Nuclear morphology of Sandimmune®-treated human PTEC. PTEC were treated for 24 h with medium (**A**) CsA 0.1 µg/ml (**B**) CsA 100 µg/ml (**C**) or the antiFas15 monoclonal antibody combined with cycloheximide (**D**) Cytospin preparations were stained with Hoechst 33258 (magnification: ×400) and examined by fluorescent microscopy. No change in nuclear morphology was seen after incubation with CsA 0.1 µg/ml. The apoptosis control (**D**) showed nuclei with characteristic signs of apoptosis, *i.e.* condensation and fragmentation (arrows). No such change was found after incubation with CsA 100 µg/ml, although nuclear morphology appeared different with some nuclei larger in size and some smaller.

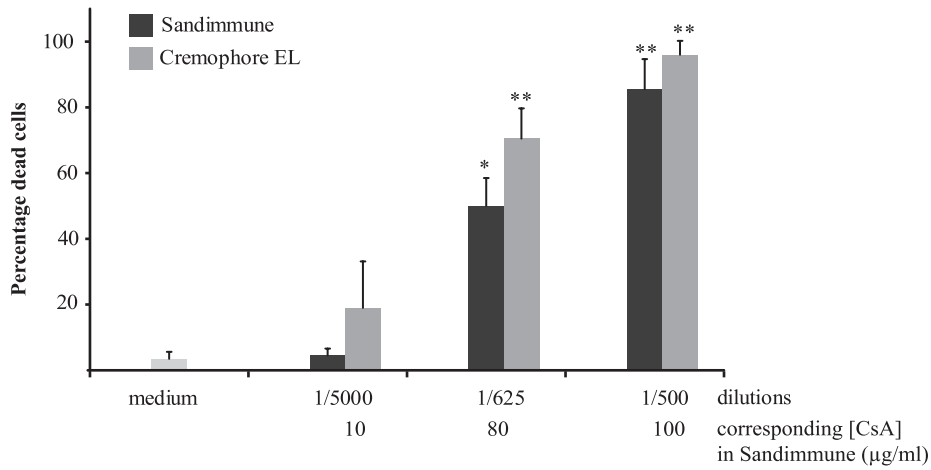
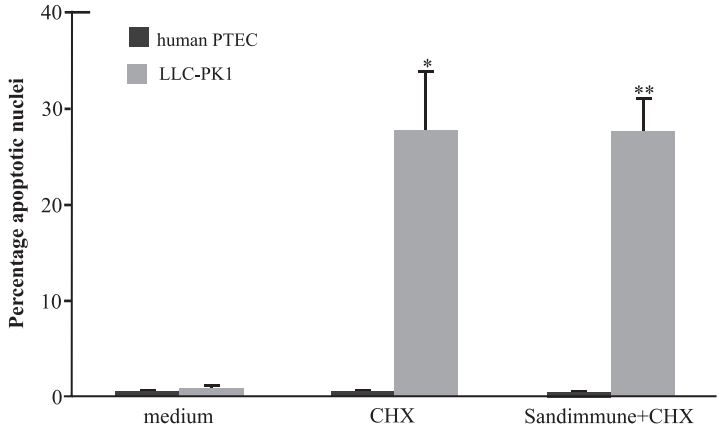


Figure 6: Comparison of the cytotoxic effect of Sandimmune® and the vehicle Cremophore EL on human PTEC. Cells were exposed to Sandimmune or vehicle at comparable dilutions for 24 h. Numbers on the *x* axis indicate the corresponding CsA concentration of the Sandimmune dilutions. Results are expressed as the mean ± SD of experiments performed in duplicate wells ($n = 3$). *$P < 0.05$ and **$P < 0.01$ compared with the medium control.

The effect of combined CsA and cycloheximide on apoptosis in primary isolates of human PTEC and LLC-PK$_1$ cells

The addition of cycloheximide may relief the resistance to a pro-apoptotic stimulus in PTEC,[12] therefore we examined the effect of CsA plus cycloheximide on both human PTEC and the porcine proximal tubular cell line LLC-PK$_1$, for which a pro-apoptotic influence of CsA has been described.[7] Human PTEC or LLC-PK$_1$ were incubated with 1 µg/ml of the clinical formulation Sandimmune combined with cycloheximide (10 µg/ml) or cycloheximide alone for 24 h, and apoptosis was evaluated by nuclear morphology (Figure 7A). In human PTEC, the addition of cycloheximide did not increase the number of cells with apoptotic nuclear morphology, whereas the combination produced apoptosis in 27.5 ± 3.5% of LLC-PK$_1$ cells. Cycloheximide alone, however, induced a comparable degree of apoptosis, disclosing a difference in the regulation of apoptosis between primary isolates of human PTEC and LLC-PK$_1$ cells. Similar results were obtained for PTEC when CsA concentrations of >10 µg/ml were used. A dose-response curve of CsA, with or without 10 µg/ml cycloheximide added to the medium, did not reveal pro-apoptotic features of CsA in LLC-PK$_1$ cells (Figure 7B). Depriving LLC-PK$_1$ cells of serum for 24 h did not change the results, and neither did treatment with CsA dissolved in alcohol. The

addition of 20% (v/v) serum to medium of human PTEC did not prime these cells to enter apoptosis during simultaneous exposure to CsA and cycloheximide (data not shown).



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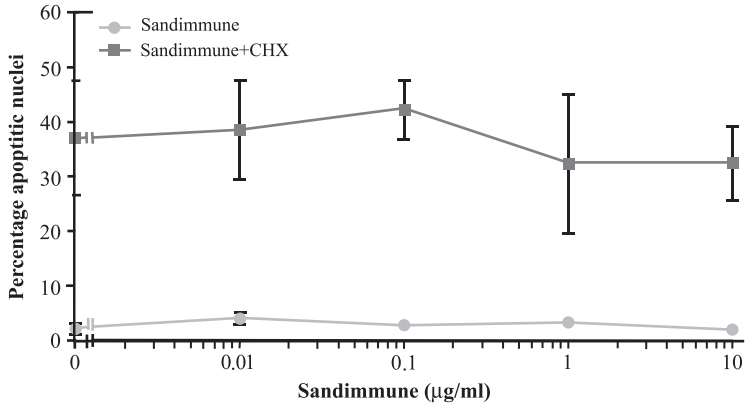

B

Figure 7: The effect of Sandimmune® combined with cycloheximide on apoptosis of human PTEC and LLC-PK$_1$ cells (**A**) Human PTEC and LLC-PK$_1$ cells were treated for 24 h with cycloheximide (10 µg/ml) with or without Sandimmune® 1 µg/ml, and apoptosis was evaluated by examining nuclear morphology. (**B**) Dose-response curve of 24-h Sandimmune® treatment of LLC-PK$_1$ in the presence of cycloheximide. In human PTEC, the addition of cycloheximide to Sandimmune® did not result in an increase in the number of cells entering apoptosis. In LLC-PK$_1$ cells, cycloheximide treatment produced a significant increase in apoptosis. However, no effect of CsA either alone or in combination with cycloheximide was noted. Results are expressed as the mean ± SD of experiments performed in triplicate wells (n = 3) (A), or the mean ± SEM of experiments performed in duplicate (n = 3) (B). *P < 0.05; **P < 0.01.

The effect of CsA on cell viability during chemical ATP depletion

Due to the renal vasoconstrictor potential of CsA *in vivo* and the demonstrated direct inhibitory effect of the drug on the ATP production of isolated mitochondria,[3,13] we decided to examine cell death of human PTEC that were cultured under simulated hypoxic conditions and co-exposed to CsA. PTEC were therefore subjected to chemical ATP depletion using glucose-free culture medium, to which 2 µM Antimycine A and 5 mM 2-deoxy-D-glucose were added. The viability of cells was assessed by the trypan blue exclusion assay and apoptosis was evaluated by examining nuclear morphology. After 3, 6 and 24 h of ATP depletion a significant increase in the number of dead cells was observed. The amount of cell death, however, was not influenced by the addition of CsA 1 µg/ml (Figure 8). No increase in apoptosis was noted at any time point or condition (data not shown). The use of a CsA concentration of 10 µg/ml did not change the results.

Discussion

In this study we examined the influence of CsA on the viability of cultured proximal tubular epithelial cells by measuring cell death through either necrosis or apoptosis. Human cells derived from primary isolates and an immortalized porcine cell line were used. Because CsA may be concentrated in renal tissue *in vivo,*[14] and the corresponding levels *in vitro* have not been determined conclusively, we also examined concentrations that appear supraphysiological (up to 100 µg/ml). The results show that when CsA is used at concentrations as seen *in vivo* there is no effect on cell viability. Also, preconditioning for apoptosis by either ATP depletion or co-treatment with cycloheximide did not reveal any pro-apoptotic activity of CsA. At very high concentrations of CsA (>10 µg/ml), as used in the clinical formulation of Sandimmune, cultured human PTEC die as a result of necrosis due to vehicle toxicity.

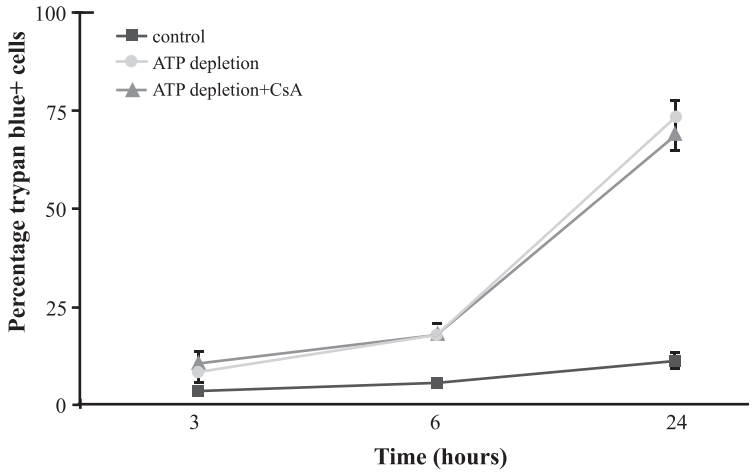

Figure 8: The effect of chemical ATP depletion and Sandimmune® treatment on the viability of human PTEC. Cells were exposed for 3, 6 and 24 h to Antimycine A (2 µM) and 2-deoxy-D-glucose 5 mM with or without Sandimmune® 1 µg/ml dissolved in glucose free culture medium. Cell death was measured by the trypan blue exclusion assay. Results are expressed as the mean ± SEM of experiments performed in triplicate wells (n = 5).

The exact pathogenesis of chronic CsA nephrotoxicity has remained elusive.[1] Histopathological studies have suggested a toxic effect of the drug on afferent arterioles and tubular epithelial cells, as exemplified by hyaline changes in these vessels, morphological alterations in proximal tubular epithelial cells and a higher rate of tubular apoptosis assessed by the TUNEL assay.[4,8] Evidence has also been presented to show that CsA may directly stimulate various cells in the kidney to locally produce profibrogenic growth factors.[15]
Whether human PTEC are a direct target for CsA toxicity remains controversial. In the past, seemingly contradictory results have been obtained using cultured tubular epithelial cells and CsA concentrations achieved *in vivo*. Two studies reported loss of viability of cultured human PTEC after CsA exposure at CsA concentrations of 0.05 or 1 µg/ml,[16-17] whereas another study did not, despite the fact that higher drug concentrations (up to 10 µg/ml) were used.[18] This variance might be explained by differences in the experimental protocols. In the first study,[16] PTEC were deprived of essential culture supplements before incubation with CsA, whereas in the second study[17] human PTEC were of fetal origin.
The results of our *in vitro* study do not support the hypothesis that CsA induces apoptosis of human PTEC directly, as no increase in apoptosis was found over the full range of CsA concentrations tested (0.01-100 µg/ml). These findings are at variance with three reports

that examined apoptosis induced by low concentrations of CsA in unspecified human tubular epithelial cells,[19] pig proximal tubular epithelial cells[20] or LLC-PK_1 cells.[7] The reason(s) for these discrepant results are not yet clear. Two of these previous studies[19] examined primary isolates of tubular cells, but used serum in their culture media.[20] In contrast, we did not add serum during the isolation or subculture period of human PTEC in order to prevent undesired outgrowth of non-tubular cells.[10] Differences in the primary cells studied may also be responsible for the variance. In another set of experiments we incubated human PTEC with tacrolimus, for which a similar histopathological pattern of nephrotoxicity has been described as for CsA. Likewise, no loss of cellular viability could be found, with 5 μg/ml the highest concentration tested (data not shown).

To relief resistance to pro-apoptotic stimuli, cycloheximide has been used successfully in cell culture systems in the past.[12] For PTEC, Fas ligation alone is not sufficient to induce apoptosis, but in combination with cycloheximide, apoptosis is readily detectable.[12] In our study, co-treatment of CsA and cycloheximide did not unmask a putative pro-apoptotic influence of CsA. Interestingly, we found a difference in regulation of apoptosis between primary isolates of human PTEC and the LLC-PK_1 cell line. In LLC-PK_1 cells, treatment with cycloheximide alone resulted in an increase in the number of cells entering apoptosis. This indicates that LLC-PK_1 cells are a less suitable model for human PTEC when apoptosis is studied.

In the present study we also decided to analyze the effect of CsA on PTEC that were chemically depleted of ATP, because CsA induces renal vasoconstriction *in vivo* and has an inhibitory effect on the ATP production of isolated mitochondria.[13] We demonstrate that CsA does not affect cell death of ATP-depleted cultured human PTEC. However, this does not exclude the possibility that *in vivo* tissue hypoxia due to vasoconstriction or obstruction of afferent renal arterioles is still responsible for tubular cell apoptosis during CsA treatment, as has been suggested by the results of a study in salt-depleted rats.[9] In this study, CsA treatment produced an increase in apoptosis in tubular cells, which was partially reversed by co-treatment with losartan, an angiotensin II type 1 receptor antagonist, or with L-arginine, a substrate for nitric oxide synthetase.

We conclude that the cellular viability of cultured adult human PTEC is not influenced by short-term exposure to CsA at physiological concentrations during normo-oxic or simulated hypoxic experimental conditions. At very high drug concentrations, cultured human PTEC die as a result of cell necrosis, an effect that might solely be based on vehicle toxicity.

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Chapter 4

Conversion from cyclosporine to azathioprine at 3 months reduces the incidence of chronic allograft nephropathy

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Abstract

Background: Conversion from cyclosporine to azathioprine after renal transplantation has been shown to be beneficial in terms of allograft function, cardiovascular risk factor profile and the incidence of gout. A higher incidence of acute rejection, however, has also been reported and uncertainty still exists about the long-term outcome after conversion. We report on the extended follow-up of an open-label, randomized trial that examined conversion to azathioprine as early as 3 months after transplantation.

Methods: One hundred twenty-eight patients were enrolled in this single-center study. Three months after transplantation they were randomly assigned to continue cyclosporine treatment ($N = 68$), or they were converted to azathioprine ($N = 60$). The steroid dose was temporarily increased in the patients who were converted.

Results: Patient survival was not different in the two groups. Graft survival tended to be lower (64.7 % *vs.* 76.5 % at 15 years) in the cyclosporine continuation group ($P = 0.14$), when data were analyzed on an intention to treat basis. The graft survival of the patients that stayed on their assigned treatment was significantly higher in the azathioprine arm, starting at two years' post-transplantation. The glomerular filtration rate was significantly higher in the patients who were converted to azathioprine. More allograft biopsies were taken from patients remaining on cyclosporine for suspicion of cyclosporine-related nephrotoxicity and prompted a high rate of late conversions (19%). The relative risk of chronic allograft nephropathy was significantly higher in the group that continued cyclosporine [relative risk 4.3, (95% CI, 1.4 to 12.9); $P = 0.009$]. Conversion to azathioprine reduced the need of blood pressure and lipid lowering drugs.

Conclusion: Conversion to a calcineurin inhibitor-free immunosuppressive regimen three months after renal transplantation improved allograft function, reduced the need of cardiovascular risk factor-controlling medication, and reduced the incidence of chronic allograft nephropathy.

Introduction

The introduction of cyclosporine A in clinical renal transplantation has improved the efficacy of immunosuppressive treatment, leading to a decline in the incidence of acute rejection episodes and allograft loss in the first year after transplantation. However, the

drug has many toxic side effects: it raises the blood pressure and serum cholesterol level and could cause nephrotoxicity, both of which contribute to mortality and graft loss in the late post-transplantation period.[1] Reducing the exposure to the drug after the period with the highest risk of acute rejection could, therefore, be advantageous. It was hypothesized that the withdrawal of cyclosporine and the replacement by azathioprine, or more recently, mycophenolate, after a certain post-transplantation time frame would preserve the better short-term results of cyclosporine therapy, while avoiding the consequences of long-term exposure to the drug. As a result, a number of clinical trials have examined the safety of cyclosporine withdrawal after renal transplantation. Improvements in renal function, lipid profile, hypertension, and the incidence of gout were reported in these conversion trials. Although a meta-analysis of the azathioprine conversion trials reported an 11% [95% confidence interval (CI), 7 to 15] higher incidence of acute rejection after conversion, these episodes were not associated with a higher rate of subsequent graft loss.[2]

Conversion or discontinuation of cyclosporine is still not common practice in most transplant centers, mainly because of uncertainty about long-term outcome. The reported follow-up of patients in the published studies has thus far been relatively short. Only two studies reported data on patient and graft survival beyond six years' post-transplantation with a maximum follow-up of 10 years.[3-4] In both studies, graft survival curves tended to deviate after five years in favor of azathioprine, but the number of patients at risk was rather limited. Therefore, it was hypothesized that after a longer period of follow-up a significant difference would occur.

An open-label prospective randomized trial was initialized at our center in 1983 that compared cyclosporine continuation with conversion to azathioprine three months after transplantation.[3] In 1995, data on 8 years' follow-up were published; in the present report we extend the analysis of outcome to a 15-year follow-up period and provide additional data on histological abnormalities seen in allograft biopsies taken during the observation period.

Methods

Patients and trial design

The design of this open-label randomized study has been previously reported.[3] In summary, a total of 128 patients were enrolled in the study. Immediately after transplantation each patient received cyclosporine and prednisone according to a standard schedule.[3] No prophylactic therapy with poly- or monoclonal T-cell antibodies was given. At 3 months'

post-transplantation, patients were randomly assigned to continue cyclosporine treatment (N = 68), or were converted to azathioprine (N = 60). For patients who continued cyclosporine treatment, the dose after randomization was 5 mg/kg daily with further dose adjustments made according to whole blood trough-level monitoring.[3] Patients who were converted to azathioprine treatment received azathioprine in a dose that was gradually increased to 2 to 2.5 mg/kg daily, depending on leukocyte count. Concomitant with the conversion, the dose of prednisone was temporarily increased and subsequently tapered slowly over a period of 10 months to a steady dose of 10 mg/day.[3] First and third acute rejection episodes were treated with high-dose corticosteroids. Second rejection episodes or steroid-resistant acute rejection episodes were treated with rabbit antithymocyte globulin. In the analysis of the study reported in 1995, acute rejection was scored as the institution of anti-rejection treatment.[3] A higher, but not statistically significant, incidence of acute rejection episodes after conversion to azathioprine was found [difference, 10.8% (95% CI, –0.2 to 22)].

For the present extended follow-up, blood pressure values, number of antihypertensive drugs, serum cholesterol levels, lipid-lowering therapy, and glomerular filtration rate (GFR) as estimated by Nankivell's formula,[5] were recorded at 3 months, 1, 5, 10, and 15 years after transplantation. The cyclosporine dosing was recorded at 1 year post-transplantation.

In addition, cardiovascular events (cardiac, cerebral or peripheral), the occurrence (and type) of cancer, and the cause of death were documented. We also evaluated the number of out-of-protocol conversions to a non-calcineurin inhibitor-based immunosuppression and the number of patients who returned to cyclosporine therapy, as well as the reasons for these changes.

Finally, the number and indication of every allograft biopsy was assessed. The following clinical guidelines were used to perform a percutaneous allograft biopsy: suspicion of acute rejection, suspicion of recurrent/de novo glomerulopathy as suggested by urinalysis; persistent isolated proteinuria >1 g/24 hr; or a gradual loss of graft function (>20% over baseline) within the observation period without an obvious explanation (*e.g.*, transplant artery stenosis or graft hydronephrosis). All pathology reports of allograft biopsies taken during the observation period were reviewed. Biopsy samples classified as chronic allograft nephropathy (CAN) were re-examined by a pathologist blinded for the instituted immunosuppressive regiment and scored according to the Banff 97 working classification of renal allograft pathology.[6] In this study, CAN was defined by functional and histologic criteria. A gradual loss of graft function (>20% over baseline) without an obvious explanation was required in combination with biopsy findings of nonspecific pathology, such as glomerulosclerosis, tubular atrophy and interstitial fibrosis with or without peripheral nodular arteriolar hyaline changes, arterial intimal fibrosis, or signs of allograft glomerulopathy.

Statistical analysis

The analysis was performed on an intention-to-treat basis. Patient survival, graft survival and the incidence of CAN were also analyzed separately in the patients who were not converted from their initial study assignment. Differences between therapy groups regarding continuous numerical variables were analyzed using the independent samples *t* test. Differences between ordered categorical variables were compared by the Mann-Whitney U test. Survival and occurrence rates were estimated by the Kaplan-Meier product limit method and factor levels compared by the Wilcoxon-Gehan test. Cox proportional hazard regression was used for estimation of relative risks. In order to allow relative risk between therapy groups to change over follow-up time, extended Cox regression was used, with therapy groups defined as time-dependent variables. Statistical analysis of the data was performed using SPSS version 10.07 (SPSS, Inc., Chicago Il, USA).

Results

Patient characteristics

The characteristics of the study populations are summarized in Table 1. No significant differences were observed.[3] The mean follow-up time of the 68 patients who were randomized to continue cyclosporine treatment was 15.2 years (range, 13.0 to 17.6), and of the 60 patients who were converted to azathioprine, mean follow-up time was 15.4 years (range, 12.8 to 18.0).

Table 1. Baseline characteristics of patients and determinants of graft function and survival

	Cyclosporine group (*N* = 68)	**Azathioprine group (*N* = 60)**
Male/female	44/24	35/25
Age *years*	43.1 (11.9)	46.1 (10.9)
First/second transplantation	59/9	54/6
Mismatches		
HLA-A: zero/one/two	33/34/1	24/34/2
HLA-B: zero/one/two	19/45/4	17/39/4
HLA-DR: zero/one/two	38/26/4	37/22/1
Highest panel reactive antibodies (%)[a]	28.1 (30.7)	30.7 (33.1)
Donor age in years [a]	34.6 (15.1)	35.9 (14.6)
Cold ischemia time *hours*[a]	29.2 (7.6)	29 (6.4)
Warm ischemia time *minutes* [a]	24.5 (5.4)	25.2 (7.5)
Number of rejection episodes		
Before randomization: 0/1/2/3	43/15/9/1	33/18/7/2
Smokers before transplantation (%)	29 (41%)	32 (53%)

HLA, human lymphocyte antigen. – [a] Mean (SD)

Adherence to treatment protocol

During follow-up, immunosuppressive therapy was changed in 15 cyclosporine-treated patients (22%) and in 16 patients who were converted to azathioprine (27%). In the cyclosporine group, the main reason for out-of-protocol switch was cyclosporine nephrotoxicity (*N* = 13, 87%), based on biopsy findings in 11 patients (84%); therapy was changed because of the suspected cyclosporine-related nephrotoxicity at a mean ± SEM of 8.6 ± 1.4 years' post-transplantation. Conversion to azathioprine was performed in 7 patients, conversion to mycophenolate in 5, and in 1 patient a lower cyclosporine trough level was pursued. Non-renal toxic side effects (gout and hypertension) were a reason to convert to azathioprine in 1 patient and to accept lower cyclosporine trough levels in another.

The main reason to change therapy in the azathioprine group was non-renal toxicity (*N* = 11, 69%). Severe side effects on liver and bone marrow were documented in 9 patients (82%). Nine patients were reconverted to cyclosporine therapy and 2 were switched to mycophenolate. One patient returned to cyclosporine treatment because of chronic rejection. In 1 patient immunosuppressive therapy was stopped because of post-

transplant lymphoproliferative disease. In 2 patients prednisone was withdrawn because of steroid- related side effects and cyclosporine was reintroduced to maintain adequate immunosuppression. The remaining patient was reconverted to cyclosporine to control a nephrotic syndrome due to recurrent glomerular disease. The reintroduction of cyclosporine therapy in 13 patients (22% of total) took place at a mean ± SEM of 4.6 ± 1.0 years' post-transplantation.

Patient survival, vascular events, cancer and causes of death

Patient survival was not significantly different in the two groups of patients. Ten and 15 years after transplantation, patient survival was 72.1% and 57.4% in the cyclosporine group versus 73.3% and 54.8% in the azathioprine group, respectively ($P = 0.93$) (Figure 1). The outcome did not differ if the analysis was restricted to patients that stayed on their initial immunosuppressive medication. Cardiovascular mortality 15 years after transplantation was also not significantly different among the two study groups (cyclosporine continuation, 21.2%; azathioprine conversion, 23.3%). Twenty patients (42.2%) in the cyclosporine group and 17 (36.2%) in the azathioprine group experienced at least one vascular event (cardiac, peripheral or cerebral) ($P = 0.57$). No difference was found in the cumulative incidence of skin cancer after 15 years (cyclosporine group, 15.2%; azathioprine group, 16%; $P = 0.5$) or cancer of other organs (9.7% *vs.* 17.7%, respectively; $P = 0.55$).

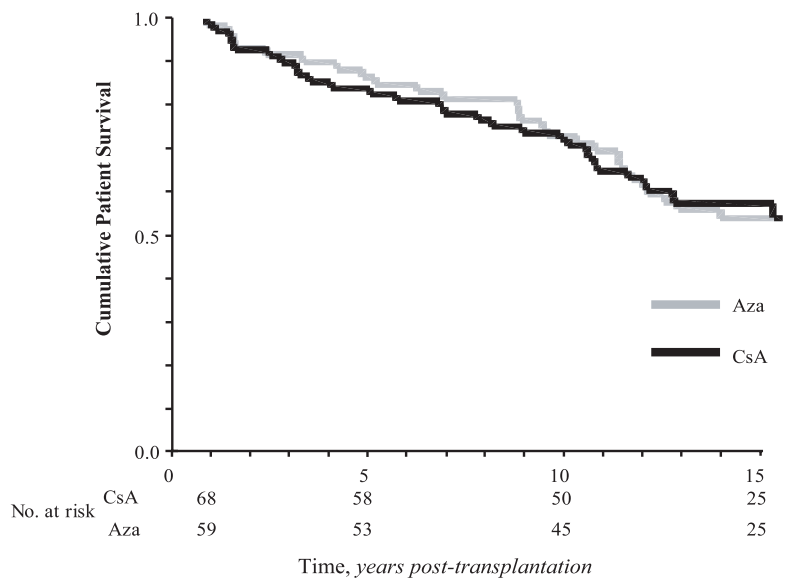

Figure 1. Patient survival.

Table 2: Effects on blood pressure, number of antihypertensive drugs required, serum cholesterol, and lipid-lowering therapy

Time post-transplantation	0		3 months		1 year		5 years		10 years		15 years	
	C	A	C	A	C	A	C	A	C	A	C	A
Systolic blood pressure	*N* = 68	*N* = 57	*N* = 65	*N* = 59	*N* = 65	*N* = 57	*N* = 46	*N* = 46	*N* = 37	*N*= 38	*N* = 16	*N* = 17
Mean *mm Hg*	147	153	144	145	144	140	148	144	144	143	137	135
Difference (95% CI)	6 (-3.5 to 15.5)		0.5 (-6.2 to 7.2)		4 (-2.4 to 10.4)		3.8 (-3.8 to 11.4)		1.5 (-8.1 to 11.2)		1.6 (-13.2 to 16.3)	
Diastolic blood pressure												
Mean *mm Hg*	87	91	89	88	89	85	89	86	85	83	83	79
Difference (95% CI)	4 (0.3 to 7.7)		0.6 (-2.7 to 3.9)		3.5 (0.3 to 6.7)		3.4 (-3.8 to 11.4)		1.9 (-2.6 to 6.4)		4.0 (-1.5 to 9.4)	
Number of antihypertensive drugs	*N* = 67	*N* = 55	*N* = 64	*N* = 58	*N* = 65	*N* = 57	*N* = 47	*N* = 46	*N* = 37	*N* = 38	*N* = 16	*N* = 17
None	47	41	17	16	12	22	6	16	2	13	0	5
One or two	18	13	42	41	45	32	35	26	25	24	11	9
Three or more	2	1	5	1	8	3	6	3	10	1	5	3
Difference (95% CI)[a]	5% (-12 to 20)		1% (-15 to 17)		20% (4 to 36)		22% (5 to 39)		29% (11 to 46)		29% (5 to 54)	
Serum cholesterol	*N* = 56	*N* = 47	*N* = 54	*N* = 44	*N* = 60	*N* = 49	*N* = 46	*N* = 44	*N* = 37	*N* = 38	*N* = 14	*N* = 17
Mean *mg/dL*[b]	255	259	259	263	282	278	271	263	228	236	209	220
Difference (95% CI)	0.1(-0.7 to 0.9)		0.1 (-0.5 to 0.7)		0.1 (-0.7 to 0.9)		0.2 (-0.5 to 0.9)		0.2 (-0.7 to 0.3)		0.3 (-1.0 to 0.3)	
Lipid lowering therapy	*N* = 67	*N* = 55	*N* = 64	*N* = 58	*N* = 65	*N* = 57	*N* = 47	*N* = 46	*N* = 37	*N* = 38	*N* = 16	*N* = 17
No	67	55	64	58	65	67	43	45	16	26	7	10
Yes	- -		- -		- -		4	1	21	12	9	7
Difference (95% CI)							6% (-3 to 16)		25% (3 to 48)		15% (-21 to 51)	

Abbreviations are: C, continued on cyclosporine; A, converted to azathioprine; CI, confidence interval.

[a] One or more vs. no antihypertensive drugs

[b] When converting to International System of Units (SI), multiply by 0.02586

Cardiovascular risk factors

Before transplantation, diastolic blood pressure was 4 mm Hg (95% CI, 0.3 to 7.7) higher in the azathioprine group (Table 2); at randomization, no significant differences in systolic or diastolic blood pressure were found between the two groups. Nine months after randomization diastolic blood pressure was 3.5 mm Hg lower in azathioprine group (95% CI, 0.3 to 6.7). No difference in blood pressure was observed at other post-transplantation intervals. At one year, however, 20% more cyclosporine-treated patients needed antihypertensive medication (95% CI, 4 to 36), and this proportion increased to 29% during follow-up (Table 2). No significant differences were found in serum cholesterol levels over time, but more patients in the cyclosporine group needed lipid-lowering therapy, which was only significant at 10 years (Table 2).

Graft survival

Graft survival censored for death with a functioning graft at 10 and 15 years was 75.3% and 64.7%, respectively, in the cyclosporine group *versus* 85.4% and 76.5%, respectively, in the azathioprine group ($P = 0.14$) (Figure 2). Although the study was not designed to detect differences in late graft loss, the graft survival curves tended to deviate with time. Therefore, we divided the follow-up time in different periods: <2 years', 2 to 5 years', and >5 years' post-transplantation. The relative risk (RR) of graft loss in the cyclosporine group compared to the azathioprine group increased with time – RR in the first 2 years of follow-up, 0.88 (95% CI, 0.22 to 3.50), $P = 0.85$; RR between 2 and 5 years follow-up, 1.54 (95% CI, 0.37 to 6.46), $P = 0.55$; and RR beyond 5 years of follow-up, 2.90 (95% CI, 0.92 to 9.07), $P = 0.070$. When we analyzed the outcomes restricted to those who were not converted from their initial assignment we found a significantly better graft survival in the azathioprine group starting at two years' post-transplantation; graft survival censored for death with a functioning graft at 2, 5, 10 and 15 years was 94.7 *vs.* 93.7%, 92.7 *vs.* 85.2%, 90.0 *vs.* 74.1%, and 81.9 *vs.* 69.2%, respectively ($P = 0.012$) (Figure 3). The RR of graft loss after two years' post-transplantation in the cyclosporine group compared to the azathioprine group was 3.23 (95% CI, 1.05 to 9.94; $P = 0.013$); the RR of graft loss in the first two years did not differ between the groups and was 1.16 (95% CI, 0.26 to 5.18; $P = 0.90$).

In both treatment arms the major cause of graft loss was death with a functioning graft: 22 patients (32%) in the cyclosporine group *versus* 23 (38%) in the azathioprine group.

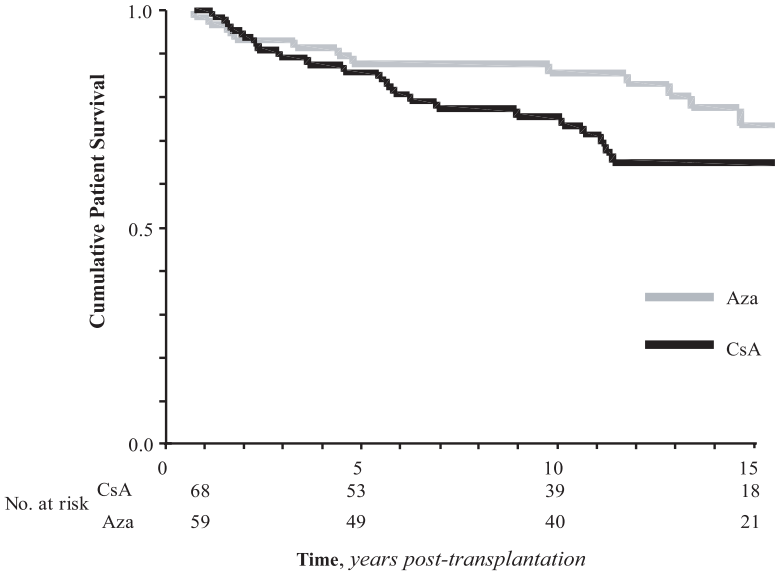

Figure 2: Graft survival censored for death with a functioning graft.

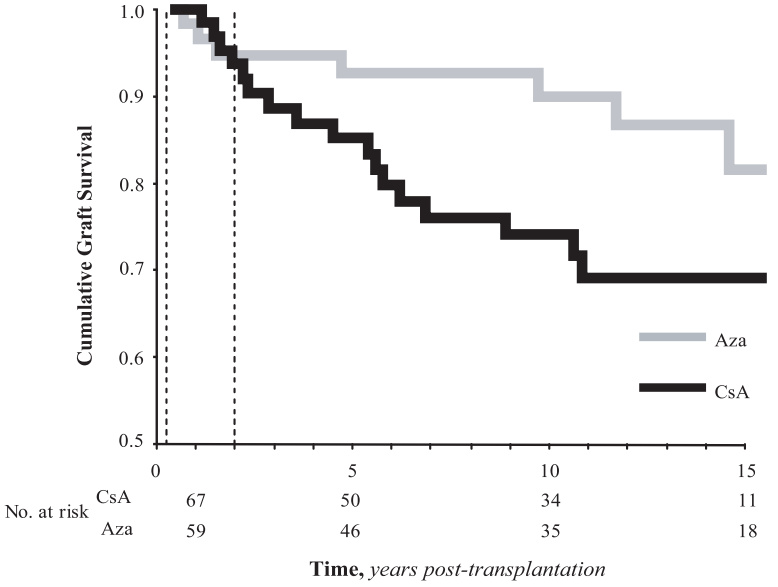

Figure 3: Graft survival censored for death with a functioning graft of the patients that stayed on the drug to which they were initially assigned. Vertical dashed lines indicate the start of the study 3 months' post-transplantation (left lines), and the start of the period of survival benefit in the azathioprine arm of the study 2 years' post-transplantation (right lines).

A gradual loss of function resulting in graft failure was seen in 18 patients (26%) who continued cyclosporine. In 16 of these patients a biopsy was performed that showed chronic allograft nephropathy (CAN) in 12 (75%), and recurrent renal disease in 4 patients (25%). In contrast, only 8 patients (13%) in the azathioprine group developed graft failure due to a gradual decline in function. Six patients were biopsied, showing CAN in 4 (67%) and recurrent renal disease in 2 patients (33%). Acute rejection resulted in graft loss in 2 patients that continued cyclosporine (3%) *versus* in 3 (5%) that switched to azathioprine treatment. In one patient in the azathioprine group, graft artery thrombosis, and in another patient, the cessation of immunosuppressive treatment because of post-transplant lymphoproliferative disease, resulted in loss of the graft.

Graft function and proteinuria

Glomerular filtration rate as estimated by Nankivell's formula, was significantly better in the patients who were converted to azathioprine (Table 3). Three months after conversion the difference measured 10.4 ml/min (95% CI, 5 to 15.9 ml/min), increased to 17.1 ml/min (95% CI, 11.6 to 22.7) at 9 months after conversion, and persisted throughout the follow-up. No significant difference between the groups in the proportions of patients with proteinuria over 1 g/24 hr was found at any point in time.

Cyclosporine dosing

The mean dose of cyclosporine taken at one year post-transplantation in the cyclosporine arm of the study measured 5.1 ± 1.4 mg/kg. The cyclosporine dosage did not differ between the patients that developed CAN or lost their graft function compared to those who did not (CsA mg/kg groups: CAN yes/no, graft loss yes/no, CAN, or graft loss yes/no, mean ± SD, 5.6 ± 1.4 *vs.* 5.0 ± 1.3; 4.8 ± 1.7 *vs.* 5.2 ± 1.2; 5.3 ± 1.6 *vs.* 5.0 ± 1.2 mg/kg; $P = 0.15$, $P = 0.28$, $P = 0.61$). The mean cyclosporine trough levels at one year post-transplantation were not significantly different between the patients of the cyclosporine group who developed CAN and those who did not. The results were similar when the analysis was limited to those who stayed on cyclosporine treatment.

Table 3: Renal function and proteinuria during follow-up

Time post-transplantation	3 months		6 months		1 year		5 years		10 years		15 years	
	C	A	C	A	C	A	C	A	C	A	C	A
Estimated GFR	*N* = 68	*N* = 60	*N* = 67	*N* = 60	*N* = 66	*N* = 57	*N* = 50	*N* = 46	*N* = 37	*N* = 38	*N* = 15	*N*= 17
Mean *ml/min*	56.5	53.5	57.3	67.8	55.7	72.9	56.3	71.0	52.8	71.7	56.3	71.7
Difference (95% CI)	3.0 (-2.6 to 8.6)		10.4 (5.0 to 15.9)		17.1 (11.6 to 22.7)		14.8 (7.7 to 21.9)		19.0 (10.1 to 27.8)		15.7 (0 to 30.6)	
Proteinuria	*N* = 68	*N* = 59	*N* = 67	*N* = 57	*N* = 66	*N* = 57	*N* = 49	*N* = 46	*N* = 37	*N* = 38	*N* = 16	*N* = 17
< 1gram/day	62	56	60	51	59	49	42	38	33	30	14	15
≥ 1	6	3	7	6	7	8	7	8	4	8	1	2
difference (95% CI) ‡	4% (-5.3 to 13)		0.1% (-11 to 11)		3% (- 15 to 8)		3% (-18 to 12)		10% (-27 to 7)		5 % (- 27 to 17)	

Abbreviations are: C, continued on cyclosporine; A, converted to azathioprine; GFR, glomerular filtration rate; CI, confidence interval.
‡ ≥1 g/day *vs*. < 1/day.

Analysis of allograft biopsies

In order to evaluate the adherence to the biopsy guidelines we reassessed the indications for the biopsies that were taken in the follow-up period (Table 4). No significant differences were found between the two groups with respect to the performance of a biopsy for a specific indication. We analyzed the histopathological findings in biopsies that were obtained more than 6 months after randomization. A total number of 53 biopsies were identified, 33 in the cyclosporine group, and 20 in the azathioprine group. Forty-seven biopsies (89%) yielded adequate material according to the Banff criteria. Of these, 30 biopsies (64%) were taken in the group that continued cyclosporine and 17 (36%) in the group that switched to azathioprine (Table 5). Acute rejection was found in 2 patients of the cyclosporine group (3%) and in 3 patients of the azathioprine group (5%). Recurrent or de novo glomerulopathy was found in 8 patients in cyclosporine group (11.8%) and in 7 patients of the azathioprine group (11.7%). CAN was diagnosed in 16 patients of the cyclosporine group (23.5%), but in only 4 patients of the azathioprine group (6.4%). In the cyclosporine group 15 patients received the drug until the diagnosis of CAN. None of the patients in the azathioprine group received cyclosporine prior to the diagnosis.

The relative risks of biopsy-proven late acute rejection or recurrent/de novo glomerulopathy did not differ between the two groups. The relative risk of biopsy proven CAN during the follow-up period was significantly higher in the group that continued cyclosporine [RR 4.3 (95% CI, 1.4 to 12.9); $P = 0.009$] (Figure 4). Similar results were obtained when the analysis was restricted to patients who stayed on their initial immunosuppressive medication.

The Banff score of the biopsy samples that were diagnosed as CAN are depicted in Table 6. Eight biopsy samples in the cyclosporine group (50%) showed signs suggestive of chronic rejection (transplant glomerulopathy and/or pronounced arterial intimal fibrosis); in 5 of these peripheral nodular arteriolar hyaline changes were also found, reminiscent of cyclosporine nephrotoxicity. In another 8 biopsy specimens of the cyclosporine group, peripheral nodular arteriolar hyaline changes were seen without signs suggestive of chronic rejection. A higher score was found for peripheral nodular arteriolar hyaline change in the cyclosporine group and a higher score for the degree of intimal fibrosis in the azathioprine group (Table 6). These histological data suggested that continued cyclosporine treatment 3 months' post-transplantation did not give better protection against chronic rejection than when a switch to azathioprine was performed. The higher incidence of CAN during continued cyclosporine treatment is most likely explained by additional cyclosporine-related renal structural changes.

Table 4: Clinical indications and number of biopsies

	Cyclosporine group		Azathioprine group		
Indication	No. %	No. biopsies performed %	No. %	No. biopsies performed %	*P* value[a]
Suspicion of acute rejection	12 (25%)	11 (92%)	11 (32%)	11 (100%)	1.000
Persistent proteinuria	4 (8%)	4 (100%)	7 (21%)	6 (85%)	1.000
Recurrent or de novo glomerulopathy	5 (10%)	5 (100%)	3 (9%)	3 (100%)	1.000
Chronic transplant dysfunction	27 (56%)	24 (88%)	13 (38%)	10 (77%)	0.370
Total	48 (100%)	44 (92%)	34 (100%)	30 (88%)	0.713

[a] For the difference in biopsy performance between the cyclosporine and the azathioprine group.

Table 5: Biopsy results in samples taken more than six months post-randomization

Biopsy diagnosis	Cyclosporine group		Azathioprine group	
	No. biopsies %	No. patients %	No. biopsies %	No. patients %
Acute rejection	3 (10%)	2 (3%)	3 (18%)	3 (5%)
Glomerulopathy (recurrent/de novo)	11 (37%)	8 (11.8%)	9 (53%)	7 (11.7%)
Chronic allograft nephropathy	16 (53%)	16 (23.5%)	5 (29%)	4 (6.4%)
Total	30	26	17	14

Table 6: Pathologic findings of biopsies diagnosed as chronic allograft nephropathy

Histological features	Cyclosporine group ($N = 16$)	Azathioprine group ($N = 4$)	*P* value
Interstitial fibrosis [a]	1.50 ± 0.16[b]	1.5 ± 0.29	0.914
Tubular atrophy [a]	1.37 ± 0.27	1.5 ± 0.29	0.768
Arterial intimal thickening [a]	1.25 ± 0.27	2.5 ± 0.29	0.039
Allograft glomerulopathy [a]	0.19 ± 0.10	0	0.360
Peripheral arteriolar hyaline change [a]	1.25 ± 0.93	0	0.017
Percentage global glomerulosclerosis	38 ± 7.2	19 ± 11.1	0.283

[a] Scored according to the Banff 97 classification on a scale of 0 to 3

[b] Mean ± SEM

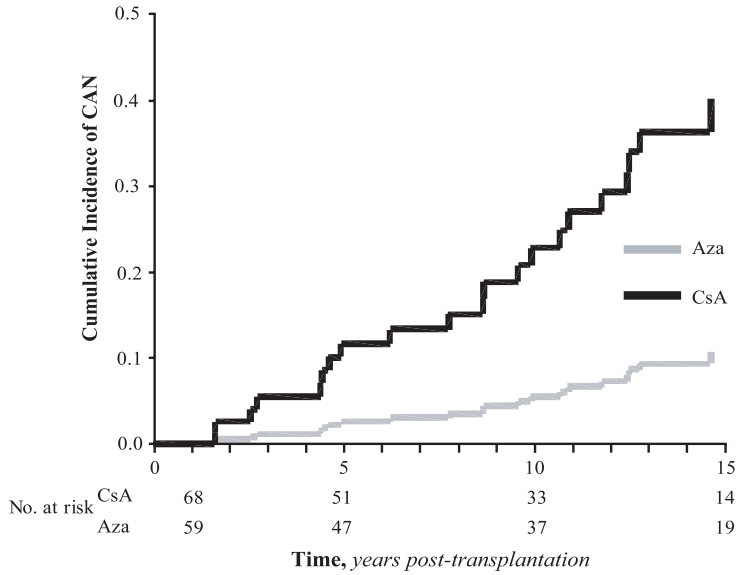

Figure 4: Cumulative incidence of chronic allograft nephropathy (CAN).

Discussion

The present paper is the first to report the results of more than 10 years' follow-up of a single-center, open, randomized trial comparing cyclosporine withdrawal and conversion to azathioprine with continued cyclosporine treatment. The conversion to azathioprine took place as early as 3 months' post-transplantation. The results show that the earlier reported beneficial effects of conversion on cardiovascular risk-factor control extend beyond 5 years' post-transplantation. Furthermore, allograft function is better preserved after conversion and a lower incidence of CAN is found.

In the intention-to-treat analysis of our study a better graft survival was observed in the azathioprine group beyond 5 years of follow-up, albeit not significant, and the relative risk of graft loss in the cyclosporine-treated patients increased with time. It has been shown that a reduction in dose or withdrawal of calcineurin inhibitors slows the rate of decline in renal function of patients with CAN.[7] The observed graft survival benefit after conversion to azathioprine in our study may have been significantly weakened by the high rate of late cyclosporine withdrawals in the cyclosporine continuation group due to cyclosporine-related

nephrotoxicity (19%). When we analyzed the outcomes restricted to those who were not converted from their initial assignment, we found a significantly better graft survival in the azathioprine group starting at two years' post-transplantation and the cumulative incidence of CAN was still significantly lower in the patients who used azathioprine. The lag time seen in the present study before the nephrotoxic effect of cyclosporine started to influence the graft survival curve confirms previous observations after conversion to azathioprine,[4] and compares well with data on cyclosporine-induced irreversible nephrotoxicity observed in cardiac allograft recipients.[8]

The estimated GFR values improved after conversion to azathioprine: three months after conversion the mean GFR was 10.4 ml/min higher. This increase can be explained by the disappearance of cyclosporine-induced renal vasoconstriction.[9] However, since the difference in GFR tended to increase further at a longer period of follow-up, these data also suggest reversibility of renal structural changes after cyclosporine withdrawal.

The pathologic changes that are found in chronic deteriorating kidney allografts are often not specific.[10] The descriptive term chronic allograft nephropathy (CAN) that has been adopted to classify these changes encompasses chronic obliterative vascular alterations, tubular atrophy, glomerulosclerosis, and interstitial fibrosis.[6] CAN includes both changes due to chronic rejection and chronic calcineurin inhibitor nephrotoxicity. Herein, we report for the first time a beneficial effect of cyclosporine withdrawal on the incidence of CAN post-transplantation. The fact that the pathology data in our study were derived from kidney biopsies performed for cause prompted us to reassess the indication and the actual performance of a biopsy. We found no difference in biopsy performance for a specific indication in the two study arms.

In our study cyclosporine trough levels were monitored routinely in the patients who continued cyclosporine, and dosing was adjusted accordingly. In spite of this, cyclosporine use had a marked influence on the incidence of CAN. The mean dose of cyclosporine taken at one year post-transplantation in the patients who continued on cyclosporine was 5.1 ± 1.4 mg/kg, which compares well with the optimal dose defined by data obtained from the Collaborative Transplant Study registry. A cyclosporine dose between 3-6 mg/kg at one year was associated with better long-term allograft survival.[11] In our study, no difference was found in cyclosporine dosage at 1 year post-transplantation between the patients of the cyclosporine group who did or did not develop CAN or who lost their transplant. Also, the mean cyclosporine trough levels at one year were not significantly different. Recently, new and better ways to monitor cyclosporine exposure of individual patients have been investigated to optimize its efficacy and to decrease toxic side effects. It has been shown that cyclosporine exposure measured by the area under the concentration over time curve (AUC) is highly variable among patients who have equal cyclosporine trough

levels.[12] Formally, we cannot exclude that a proportion of our patients in the cyclosporine continuation group was overexposed to the drug and that better monitoring of cyclosporine therapy will reduce the incidence of cyclosporine nephrotoxicity and the occurrence of CAN post-transplantation.

The finding that conversion to non-calcineurin immunosuppressive treatment reduces the long-term incidence of CAN is of special importance in an era of donor shortage, with an increasing use of kidneys from marginal donors.[13-15] The recipients of these kidneys are likely to be more prone to graft failure as a result of cyclosporine nephrotoxicity than the recipients of a regular allograft merely because they cannot tolerate some loss of function.[16]

The single most important cause of graft loss in both treatment arms of our study was death with a functioning graft, which was usually related to a cardiovascular event.[17-21] Renal transplant patients, after the first post-transplant year, experience a five-fold increase in cardiovascular mortality compared with age-matched controls.[19] This stresses the need to reduce cardiovascular risk factors both before and after transplantation. The withdrawal of cyclosporine and replacement by azathioprine 3 months after transplantation reduced the need of cardiovascular risk-controlling medication over the entire 15-year period of follow-up.

The question can be raised whether every patient should be converted to a non-calcineurin-based immunosuppressive regimen after a certain period of time. It is important to note that our study was performed in a cohort of predominantly Caucasian recipients, well matched for human lymphocyte antigen (HLA), and therefore the results cannot directly be extrapolated to other populations.[16,22-24] Furthermore, the conversion to azathioprine was not completely harmless, as substantial toxicity of azathioprine therapy was encountered. In fact, the results of this study should encourage the use of mycophenolate as the "conversion agent" of choice rather than azathioprine. Mycophenolate therapy has a better safety profile than azathioprine, has been associated with less acute rejection after conversion compared to azathioprine, and may by itself, according to U.S. renal transplant scientific registry data, further decrease the incidence of CAN.[25-29]

Conclusion

Cyclosporine withdrawal 3 months after transplantation can be done safely, reduces the need of cardiovascular risk factor-controlling medication, improves allograft function, and reduces the incidence of CAN.

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Chapter 5

Early interstitial accumulation of collagen type I discriminates chronic rejection from chronic cyclosporine nephrotoxicity

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Abstract

Little is known regarding the composition of the interstitial extracellular matrix of kidney allografts with deteriorating function. Collagen I, III, and IV, the collagen IV α3 chain, and the laminin β2 chain were investigated in biopsies of allografted kidneys with chronic cyclosporine A nephrotoxicity (CsAT) (*N* = 17), chronic rejection (CR) (*N* = 12), or chronic allograft nephropathy (CAN) (*N* = 19). α-Smooth muscle actin expression was also examined. Normal native kidneys were used as control samples (*N* = 11). Biopsy samples were studied with routine light microscopy and immunostaining. The mean interstitial fibrosis scores were significantly higher for the CR and CAN groups, compared with the chronic CsAT group. The cortical tubulointerstitial areas of the CR and CAN groups, but not the chronic CsAT group, contained more collagen I than did normal control samples. Differences were noted even in biopsies with mild fibrosis. Accumulation of collagen III, IV and IV α3 was increased in all patient groups. Collagen III accumulation was greater in the CR and CAN groups than in the chronic CsAT group. Receiver-operating characteristic curve analysis demonstrated that collagen I staining had the best discriminatory value in differentiating CR from chronic CsAT with a sensitivity of 63% and a specificity of 94% at a cutoff value of 19%. Laminin β2 staining did not differentiate CR from CsAT. Increased α-smooth muscle actin staining did not differ among the three groups. It was concluded that, during chronic CsAT, collagen III and IV were preferentially accumulated in the tubulointerstitium. Early increases in the deposition of collagen I, with collagen III and IV, were more specific for CR. CR seems to elicit a more pronounced fibrotic response than does chronic CsAT.

Introduction

Late loss of allograft function remains a major problem in renal transplantation.[1] Kidney allograft failure is usually preceded by a process of chronic transplant dysfunction, which is characterized by a relatively slow but variable rate of decline in GFR, increasing proteinuria, and increases in BP. The pathologic changes in deteriorating grafts are often less specific. The term chronic allograft nephropathy (CAN) has been adopted to classify these changes, which include chronic obliterative vascular alterations, tubular atrophy, glomerulosclerosis, and interstitial fibrosis.[2]

CAN includes both chronic rejection (CR) and chronic calcineurin inhibitor toxicity.[2] The nature of vascular, glomerular, and interstitial changes in CAN biopsy specimens

can sometimes help to define more specifically the cause of graft dysfunction. Concentric intimal thickening of arteries and arterioles, often accompanied by a moderate degree of mononuclear cell infiltration of the vessel walls, and duplication of the glomerular basement membrane are thought to indicate chronic rejection. Smooth muscle cell necrosis and peripheral nodular medial hyalinosis of arterioles are considered hallmarks of cyclosporine A (CsA) nephrotoxicity (CsAT).[3-4]

Extracellular matrix (ECM) accumulates in the cortical interstitium regardless of the cause of chronic graft dysfunction, as observed in native kidneys with chronic diseases.[4-5] Surprisingly, the ECM composition of deteriorating allografted kidneys has rarely been studied. Determination of the ECM molecules that accumulate could enhance our understanding of the pathogenesis of graft dysfunction and could potentially help define the cause, if disease-specific changes are observed. In this study, we investigated whether the ECM composition differs between allografts that lose function because of CR and allografts with chronic CsAT. We studied the cortical interstitial ECM composition of kidney allografts of three groups of patients, *i.e.*, patients with chronic CsAT, patients with CR, and patients who were receiving CsA treatment but were most likely to experience CR.

Materials and Methods

Patient Selection

All kidney allograft biopsies that were performed in our center for clinical reasons >1 yr after transplantation, in the period between March 1, 1976, and March 1, 2001, were reviewed. Cases that had available frozen-tissue and that met the criteria defined below were included. Patients with graft artery stenosis or diabetes mellitus were excluded. In addition, biopsy samples with histologic signs of *de novo* or recurrent glomerulonephritis or acute rejection were excluded. The group designated the chronic CsAT group ($N = 17$) consisted of patients who exhibited progressive declines in allograft function only after a switch was made from a CsA formulation with a lower bioavailability (Sandimmune; Sandoz, Basel, Switzerland) to one with a higher bioavailability (Neoral; Novartis, Basel, Switzerland).[6] A brief period of acute rejection in the early posttransplantation period was allowed and occurred in eight cases. Before the switch, the patients used Sandimmune once daily, aiming at a 24-h trough level of 100 μg/liter, and demonstrated stable graft function for a mean of 5.1 ± 3.4 yr. After conversion to twice-daily Neoral therapy, a

higher target 12-h trough level of 150 µg/liter was adopted. To reach this level, the mean CsA dose was increased from 3.2 to 3.5 mg/kg.[6] Nephrotoxicity was not observed in the first months after conversion but became evident after 12 mo.[6] Biopsies were obtained a mean of 2.4 ± 1.2 yr after switching. None of the studied biopsies in this group exhibited signs of chronic allograft glomerulopathy. No positive staining for C4d in the peritubular capillaries (see below) was observed.

The patient group designated the CR group (N = 12) consisted of patients who exhibited progressive declines in renal allograft function with a calcineurin inhibitor-free immunosuppressive regimen. Immunosuppressive therapy consisted of prednisone and azathioprine. All of these patients initially demonstrated good allograft function (Table1). Thirty-three percent exhibited C4d positivity in their peritubular capillaries.

Table 1: Clinical characteristics of the patients studied[a]

Clinical Characteristic	Patient group		
	CsA Toxicity (N = 17)	CAN (N = 19)	CR (N = 12)
Time after transplantation (yr)	7.1 ± 3.3	5.5 ± 4.1	5.1 ± 4.3
Allograft age (yr)	51 ± 9	42 ± 15	34 ± 12[b]
BP, systolic (mmHg)	148 ± 19	154 ± 19	157 ± 22
BP, diastolic (mmHg)	86 ± 5	88 ± 9.7	90 ± 12
No. of antihypertensive medications	1.8 ± 1.1	2 ± 1.2	1.9 ± 1.1
Creatinine clearance (ml/min)	38 ± 12	34 ± 15	32 ± 7
Loss of clearance (ml/min)	26 ± 10	39 ± 19	49 ± 23[b]
Proteinuria (g/24 h)	1.3 ± 1.5	1.9 ± 1.9	4.0 ± 3.3
CsA trough level (µg/ml)	114 ± 30	113 ± 31	

[a] Values are mean ± SEM. CsA, cyclosporine A; CAN, chronic allograft nephropathy; CR, chronic rejection.

[b] $P < 0.05$, compared with chronic CsA toxicity and CAN groups.

A third group of patients received CsA but their allograft biopsy suggested CR as the cause of declines in allograft function. This group, designated the CAN group, consisted of patients with progressive declines in renal allograft function and biopsy findings suggesting CR, including arterial intimal fibrosis (N = 15) and/or chronic allograft glomerulopathy with glomerular basement membrane duplication (N = 4). No temporal relationship existed between the loss of function and a switch to Neoral for any of these patients. In this group, 21% of the biopsies were positive for C4d deposits in the peritubular capillaries. The

control group (N = 11) consisted of specimens from normal kidneys obtained in autopsies and from normal kidneys that had not been used for transplantation for anatomic reasons. Clinical and laboratory data were obtained in chart reviews. The collected data included posttransplantation time, allograft age (donor age plus time after transplantation), systolic and diastolic BP, number of antihypertensive medications used, creatinine clearance (as estimated with the Cockroft-Gault formula), loss of allograft function, proteinuria, and CsA trough levels at the time of biopsy. The loss of allograft function was defined as the difference in the creatinine clearance measured before the worsening of allograft function, as assessed by the breakpoint in the regression lines of 1/serum creatinine, and the creatinine clearance measured at the time of the index biopsy.[7]

Light Microscopy

After routine staining, the biopsy specimens were coded and re-evaluated with light microscopy. An experienced pathologist who was blinded with respect to the instituted immunosuppressive regimen scored the histopathologic changes. The Banff 97 classification system for histopathologic scoring of allografted kidneys was used.[8]

Immunostaining

The ECM components that were studied were detected with an indirect immunoperoxidase technique. Staining for each molecule was performed in one session. The primary antibodies used were polyclonal mouse anti-human collagen I, anti-human collagen III, and anti-human collagen IV (Harlan Sera-Lab, Sussex, UK) and monoclonal mouse antibodies against the collagen IV α3 (Wieslab, Lund, Sweden) and laminin β2 (C4) (Developmental Studies Hybridoma Bank, Iowa City, IA) chains. The secondary antibody consisted of peroxidase-coupled rabbit anti-mouse IgG (Dako, Glostrup, Denmark). Cryostat sections (4 μm) were mounted on glass slides, dried for 1 h, and stored at -20°C until used. The tissue was fixed with 4% formalin for 10 minutes. After blocking of endogenous peroxidase by incubation for 30 min with 0.1% H_2O_2 in phosphate-buffered saline, the sections were incubated for 30 min with 5% normal rabbit serum. The sections were then incubated with the primary antibody for 1 h, followed by incubation with the secondary antibody for 30 min. The sections were incubated for 10 min in a filtered solution of 0.5 mg/ml diaminobenzidine and 0.02% H_2O_2 and were then incubated for 5 min with 0.05% Cu_2SO_4 in saline solution. Counterstaining with hematoxylin was performed for 15 sec. Between all steps, the slides were rinsed with phosphate-buffered saline.

Immunofluorescence staining for C4d was performed on untreated slides, as described previously.[9] Mouse anti-C4d (Quidel, San Diego, CA) was used as the primary antibody.[9] The secondary antibody was FITC-conjugated goat anti-mouse Ig (Sigma Chemical Co., St. Louis, MO). Staining was independently evaluated by two authors (Drs. Koop and Eikmans), who were blinded with respect to the diagnoses. A previously described scoring method was used.[10]

Image Analysis

After immunostaining, the slides were coded and analyzed in a blinded manner. Digital image analysis was performed with a Zeiss microscope equipped with a full-color 3CCD camera (DXD 950p; Sony Corp., Tokyo, Japan) and KS-400 image analysis software, version 3.0 (Zeiss-Kontron, Eching, Germany). This method of digital image analysis has been validated and demonstrated to be highly reproducible.[11-12] Images of the renal cortex of each biopsy specimen were obtained and analyzed with the aid of automated script protocols (macros) developed in our department. Recording and analysis of images were performed with fixed settings. The software allowed censoring of regions of noninterest. Larger vessels and glomeruli were omitted from the analyses. The deposition of each ECM component was determined in each biopsy sample by calculation of the percentage of surface area stained by the indicator dye, relative to the total surface area. The degree of deposition was assessed by evaluation of the entire renal cortex, with a minimum of five microscopic fields per biopsy sample (at $\times$200 magnification).
The biopsy specimens that were stained for the laminin β2 and collagen IV α3 chains were also directly evaluated for interstitial or tubular expression of those molecules, by two investigators (Drs. Bakker and Koop) who were blinded with respect to the diagnosis. In the few cases of discordant scoring, decisions were reached by consensus.

Statistical analysis

Statistical analysis was performed using SPSS for Windows software (SPSS, Inc., Chicago, IL). One-way ANOVA was used for comparisons of continuous variables with normal distributions. Either a Bonferroni or Games-Howel *post-hoc* procedure was used when appropriate. The Mann-Whitney *U* test was used to compare continuous variables that were not distributed normally. The data are expressed as mean ± SD or mean ± SEM, as indicated. In a general linear model, values were adjusted for differences in the loss of

allograft function and creatinine clearance at the time of biopsy. Correlations between the degree of deposition of individual ECM components and the clinical characteristics of the patients were evaluated with the Pearson correlation test. $P < 0.05$ was considered statistically significant. Using a receiver-operating characteristic curve, we determined the cutoff point of the percentage of staining for the various ECM molecules that predicted the presence of CR with the best combination of sensitivity and specificity.

Results

Patient data

The clinical characteristics of the patients are presented in Table 1. The mean age of the allografted kidney was lower for the patients with CR, compared with the patients with chronic CsAT or CAN. The loss of creatinine clearance was greater for the patients with CR. The time after transplantation, BP values, number of antihypertensive drugs prescribed, and creatinine clearance at the time of biopsy were similar among the groups. No difference in CsA trough levels was observed between the groups receiving CsA.

Light-microscopic findings

The chronic histopathologic changes that were observed in the biopsy samples in routine light-microscopic analyses are summarized in Table 2. The mean interstitial fibrosis scores for the CR and CAN groups were significantly higher than that for the chronic CsAT group (CR group *versus* chronic CsAT group, $P = 0.011$; CAN group *versus* chronic CsAT group, $P = 0.001$). The tubular atrophy scores were higher for the CR and CAN groups, but the differences did not reach statistical significance (CR group *versus* chronic CsAT, $P = 0.422$; CAN group *versus* chronic CsAT, $P = 0.159$). The percentages of global glomerulosclerosis did not differ among the groups. The mean peripheral arteriolar hyaline thickening score for the chronic CsAT group was higher than those for the CR and CAN groups, as expected from the case definitions.

Table 2: Light-microscopic features of the allograft biopsies studied[a]

	Patient group		
Histological Feature	CSA Toxicity ($N = 17$)	CAN ($N = 19$)	CR ($N = 12$)
Interstitial fibrosis score[b]	0.58 ± 0.21	1.76 ± 0.19 [c]	1.65 ± 0.25 [d]
Tubular atrophy score[b]	0.69 ± 0.24	1.36 ± 0.22	1.29 ± 0.29
Arterial intimal thickening score[b]	0.77 ± 0.21	1.48 ± 0.19	1.40 ± 0.25
Allograft glomerulopathy score[b]	0.02 ± 0.17	0.25 ± 0.15	0.33 ± 0.19
Peripheral arteriolar hyalinosis score[b]	1.92 ± 0.12 [e]	0 ± 0.11	0 ± 0.15
Global glomerulosclerosis (%)	28.0 ± 7.1	32.5 ± 6.4	24.4 ± 8.4

[a] Values are mean ± SEM.
[b] Scored according to the Banff 97 classification system.
[c] $P < 0.01$, compared with chronic CsA toxicity group.
[d] $P < 0.05$, compared with chronic CsA toxicity group.
[e] $P < 0.01$, compared with CAN or CR group.

ECM components

First we studied the typical interstitial collagens, *i.e.*, collagens I and III. The mean areas stained for these collagens are presented in Figure 1. The collagen I-stained areas were significantly increased in the CAN and CR groups, compared with the control group, but were not increased in the chronic CsAT group (mean area ± SEM for the CAN, CR, chronic CsAT, and control groups, 18.0 ± 2.5, 23.2 ± 4.4, 6.5 ± 1.5 and 3.3 ± 0.6%, respectively). No significant difference was observed between the CAN and CR groups.

The areas stained for collagen III were increased in all patient groups (mean area ± SEM for the CAN, CR, chronic CsAT, and control groups, 26.8 ± 2.4, 28.9 ± 2.7, 16.7 ± 2.1, and 6.9 ± 2.2%, respectively). However, the areas of staining were significantly greater for the CAN and CR groups, compared with the chronic CsAT group. The staining areas did not differ significantly between the CAN and CR groups. Figure 2 presents representative images of immunostaining for collagen I and III in an allografted kidney with CR.

Next we studied whether typical basement membrane collagen type IV expression was increased to the extent observed in native kidney disease.[5] The mean areas of staining for collagen IV were significantly increased in all patient groups, compared with the control group, to the same degree (mean area ± SEM for CAN, CR, chronic CsAT, and control groups, 10.3 ± 5.1, 13.3 ± 5.6, 12.2 ± 1.4, 5.9 ± 1.5%, respectively) (Figure 3A). We also compared the staining areas for the three types of collagen in the three groups of patients.

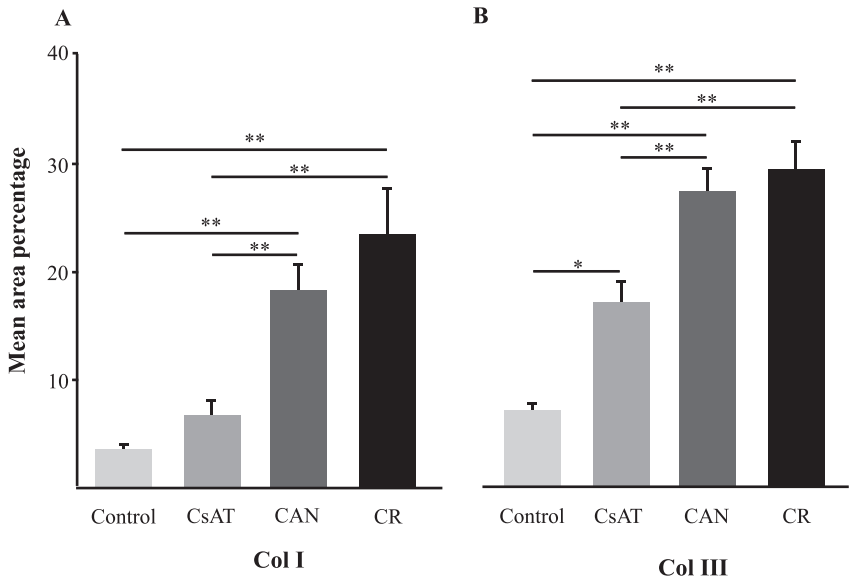

Figure 1: Staining areas (mean ± SEM) for collagen (Col) I (A) and collagen III (B). CsAT, cyclosporine A nephrotoxicity; CAN, chronic allograft nephropathy; CR, chronic rejection. * $P < 0.05$, and ** $P < 0.01$.

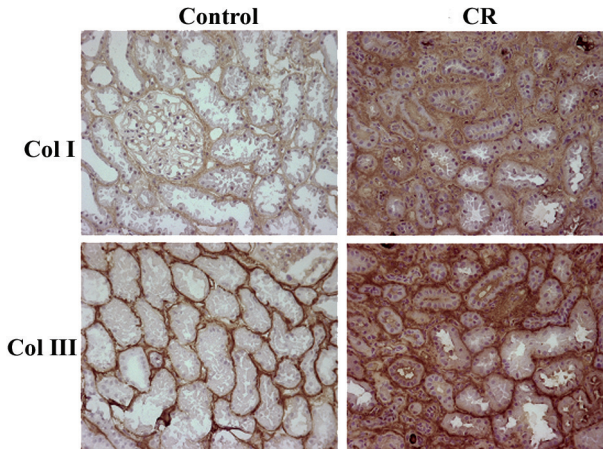

Figure 2: Immunoperoxidase staining for cortical collagen (Col) I and III. Representative images of a normal control sample (left) and a kidney allograft with CR (right) are shown.

In the CAN and CR groups, the areas staining for collagen III were greater than those for collagen IV (CAN group, $P < 0.001$; CR group, $P < 0.01$) but did not differ significantly from those staining for collagen I. In the CsAT group, the area staining for collagen III was greater than that staining for collagen I ($P < 0.001$), but no significant difference was observed in comparison with the area staining for collagen IV ($P = 0.07$).

When we studied biopsies with mild interstitial fibrosis (<25% interstitial expansion), we observed a difference in collagen I staining between the CsAT group and the CR and CAN groups combined (CsAT group, 6.5 ± 6%; CAN/CR group, 15.5 ± 8.7%, $P < 0.05$). In this subgroup with mild fibrosis, there was no difference between the time that had elapsed since transplantation (CAN/CR group) compared to the time that had elapsed since the Neoral switch (CsAT group). Collagen III and IV staining areas did not differ between the CsAT group and the CAN/CR subgroup. A meaningful comparison of biopsies with greater degrees of interstitial expansion was not possible because of the small numbers.

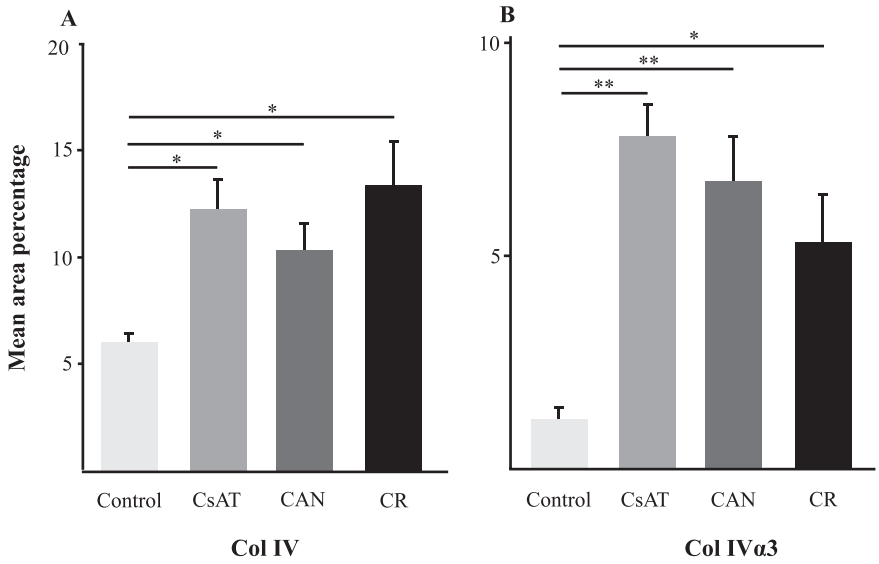

Figure 3: Staining areas (mean ± SEM) for collagen (Col) IV (A) and the collagen IV α3 chain (B). * $P < 0.05$, ** $P < 0.01$.

Because an earlier report suggested that immunostaining for the α3 chain of collagen IV and the β2 chain of laminin could differentiate CR from chronic CsAT,[13] we also evaluated these molecules. The areas stained for the collagen IV α3 chain were increased in all patient groups, compared with the control group, to the same extent (mean area ± SEM for the CAN, CR, chronic CsAT, and control groups, 6.7 ± 1.1, 5.3 ± 1.2, 7.8 ± 0.8, and 1.1 ± 0.3%, respectively) (Figure 3B). The collagen IV α3 chain was expressed in a normal pattern, namely, at the distal tubular basement membrane (TBM), in all three

patient groups. No interstitial staining or new expression at the proximal TBM was noted. In none of the groups did the areas staining for the laminin β2 chain change significantly, compared with the control group (Figure 4). However, we occasionally observed some new abnormal tubular expression in each of the groups (CsAT group, nine biopsy samples, 53%; CAN group, eight samples, 42%; CR group, six samples, 50%). The new expression was mostly confined to atrophic tubules or tubules surrounded by infiltrate, the proximal or distal morphologic features of which were difficult to assess. Less than 1% of tubules were affected in these samples, except for one sample in the CsAT group, three samples in the CAN group, and two samples in the CR group, in which 1 to 10% of tubules exhibited staining. No interstitial staining for the laminin β2 chain was observed, except for faint staining in some areas of periglomerular fibrosis.

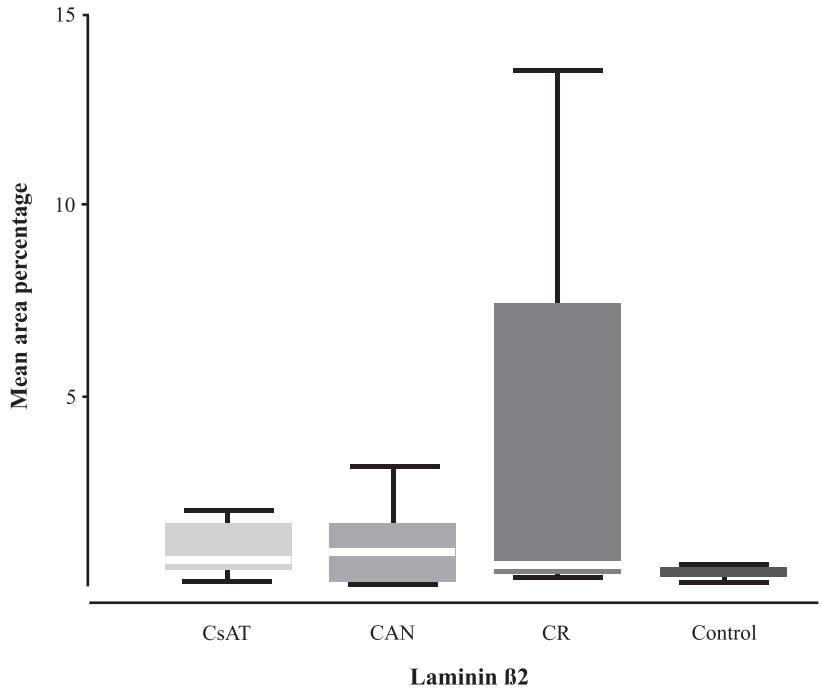

Figure 4: Box and whisker plots of the median staining areas for the laminin β2 chain. The boxes contain 50% of the values. The upper and lower borders indicate the 25th and the 75th percentiles, respectively. The upper and lower whiskers indicate the highest and lowest values, respectively. The white lines in the boxes indicate the medians. No significant differences were observed among the groups. (*P* values from the Mann-Whitney test: CsAT group *versus* CAN, CR, and control groups, 0.847, 0.925, and 0.056, respectively; CAN group *versus* CR and control groups, 0.868 and 0.172, respectively; CR group *versus* control group, 0.463).

We investigated whether the observed qualitative and quantitative differences in the interstitial ECM composition in kidneys with CR or chronic CsAT were correlated with the numbers of interstitial myofibroblasts present, as assessed with immunohistologic staining for α-smooth muscle actin (SMA). Increases in SMA staining were observed for all patient groups, compared with the control group, but no differences were observed among the patient groups (Figure 5).

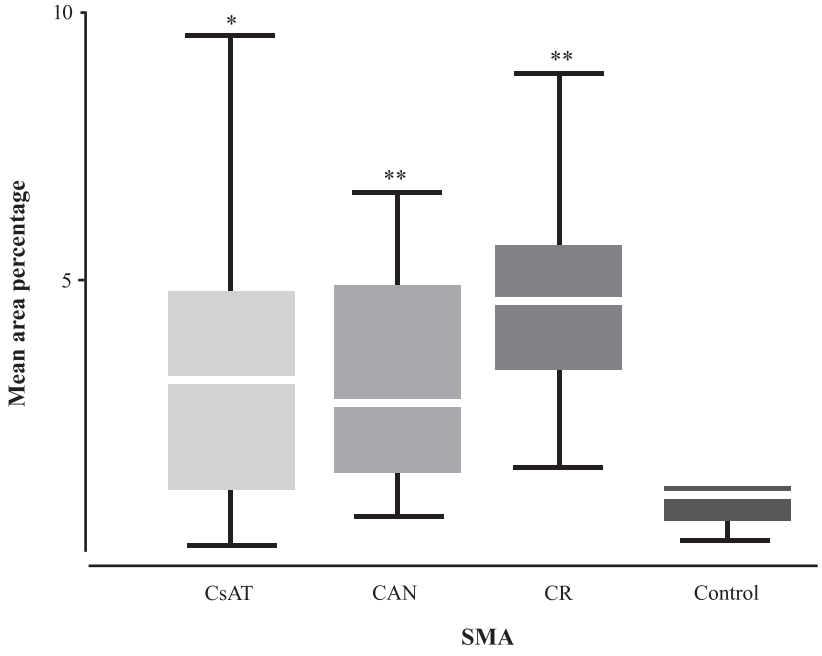

Figure 5: Box and whisker plots of the median staining areas for α-smooth muscle actin (SMA). * $P < 0.05$, ** $P < 0.01$, compared with control group.

Correlations and receiver-operating characteristic curve analysis

Among the three groups of patients, we observed no correlation between the area of staining for the various molecules and the clinical variables of loss of allograft function, time after transplantation, allograft age, creatinine clearance, and proteinuria at the time of biopsy. A positive correlation ($r^2 = 0.379$, $P = 0.008$) was observed between the interstitial fibrosis scores of all biopsy samples combined and the loss of creatinine clearance at the time of

biopsy. The interstitial fibrosis scores of all biopsy samples combined were also correlated with the areas of staining for collagen I and III (r^2 = 0.499, P = 0.001, and r^2 = 0.499, P = 0.002, respectively). A general linear model was used to adjust for differences in loss of function and creatinine clearance at the time of biopsy among the groups.

Receiver-operating characteristic curve analysis demonstrated that collagen I staining had the best discriminatory value in differentiating CR from chronic CsAT, with a sensitivity of 75% and a specificity of 88% at a cutoff value of 13% cortical interstitial staining and with values of 63% and 94%, respectively, at a cutoff value of 19% staining. The fraction of the area under the curve was 0.92 of the total area (P = 0.001). None of the ratios of the mean staining areas for the studied molecules proved to be useful for discrimination of CR from chronic CsAT.

Discussion

The aim of this study was to examine the composition of the cortical interstitial ECM of kidney allografts with either CR or chronic CsAT. We wondered whether quantitative and/or qualitative differences in ECM accumulation occur. Therefore, we quantitatively compared the deposition of several ECM molecules. We chose to study the molecules collagen I, III, and IV, because these are known to accumulate in the renal cortical interstitium during native kidney disease.[5,14] The laminin β2 and collagen IV α3 chains were investigated because an earlier report suggested that *de novo* expression of these molecules at the proximal TBM could differentiate CR from chronic CsAT.[13]

In this study, we observed quantitative and qualitative differences in the ECM composition of kidney allografts with CR or chronic CsAT. Our results demonstrated that collagen I, III, and IV accumulated in the renal cortical interstitium during CR. The most prominent increases were observed for collagen I and III. A similar pattern of ECM deposition was observed in the kidneys of patients who likely had CR but were receiving CsA. In the kidneys of patients with chronic CsAT, however, only collagens III and IV accumulated significantly.

Regulation of renal interstitial ECM composition *in vivo* is complex and encompasses changes in ECM biosynthesis and degradation. In normal kidneys, collagen I and III are observed in the blood vessels and the interstitium (albeit in small amounts) (Figure 2).[15] Collagen IV is a natural component of the glomerular basement membrane and TBM, and its α3 chain is normally observed in the basement membranes of glomeruli and distal tubules.[15] Laminin is observed in vessel walls and all basement membranes. The β2 chain, however, is observed only in glomeruli and vessel walls.[16]

Quantitative data on the ECM composition of the cortical interstitium of human allografts with chronic CsAT or CR were lacking. A few studies reported increases in collagen III in grafts with chronic dysfunction but did not compare changes induced by CR and chronic CsAT.[17-18] A number of *in vitro* studies have addressed the effects of CsA on the production of ECM molecules by renal cells. One study reported that CsA stimulated the production of pro-collagen I and IV in cultures of murine proximal tubular epithelial cells and the production of pro-collagen I in cultures of murine renal fibroblasts.[19] Increases in the cortical expression of collagen I and IV were observed *in vivo* in a rat model of CsAT.[20] The deposition of collagen III was not assessed in that model, however. Other data on collagen production were derived from a study on cultured human cells, which demonstrated an increase in collagen III production by renal fibroblasts when they were treated with CsA.[21] Studies performed with a monkey renal fibroblast cell line (CV1) demonstrated that CsA stimulated the synthesis of type III collagen by a pathway leading to activation of the *COL3A1* promoter and up-regulation of *COL3A1* mRNA.[22] We observed the increased deposition of collagen III and IV in the interstitium of the cortex of human renal allografts as a result of chronic CsAT. In contrast to the results of the study performed in rats, we observed no significant increase in collagen I deposition. Although we did not observe a correlation between the time elapsed since the Neoral switch and the degree of collagen I deposition, we cannot exclude the possibility that, with time, collagen I would also accumulate during long-term chronic CsAT. The difference in collagen I staining between the CsAT and CAN/CR subgroups with mild fibrosis suggests that CR may stimulate collagen I accumulation earlier than does CsAT.

In our study, we observed greater expansion of the interstitial space in the CR group, compared with the chronic CsAT group, as well as more deposition of collagen type III. The difference in interstitial fibrosis could not be explained on the basis of differences in clinical variables, because no correlations with those variables were observed.

We wondered whether the differences in ECM deposition between CR and chronic CsAT could be explained on the basis of a difference in the numbers of myofibroblasts present. SMA is a marker of activated myofibroblasts, which are thought to play a major role in the deposition of scar tissue.[23] Two earlier reports described increases in interstitial SMA staining in deteriorating human kidney allografts.[17-18] In CR, SMA-positive cell numbers were reported to increase with increases in the interstitial area fraction and collagen III deposition.[18] In our study, we did not observe differences in SMA staining among the CR, CAN, and chronic CsAT groups; all samples contained more SMA-positive cells than normal. To explain the greater degree of interstitial ECM deposition during CR, compared with chronic CsAT, a higher rate of collagen production by myofibroblasts or a lower rate of ECM degradation by tissue metalloproteinases could be hypothesized. Data derived

from a primate model of chronic cardiac rejection support the latter hypothesis, because it was demonstrated that the progression of myocardial fibrosis was associated with increased expression of tissue inhibitor of metalloproteinases 1 and 2.[24]

We also examined the α3 chain of collagen IV and the β2 chain of laminin. Our results are at variance with a report by Abrass *et al.,*[13] which suggested that a pattern of new expression of those molecules at the proximal TBM was specific for CR. In that study, CsAT resulted in the interstitial deposition of collagen I and III. Our study differs in the definitions of cases, the antibodies used, and the mode of analysis. The study by Abrass *et al.*[13] examined patterns of distribution and provided no quantitative data on the investigated molecules. According to Abrass *et al.,*[13] collagen I and III are not normally observed in the cortical interstitial area and the collagen IV α3 or laminin β2 chains are not expressed at the TBM. However, we and others observed that collagen I and III are normally present in the renal interstitium, albeit in small amounts.[5,15,25-27] We also observed that the collagen IV α3 chain is normally expressed at the distal TBM, as noted earlier.[16,28] This discrepancy may be related to the different antibodies used. In the study by Abrass *et al.,*[13] allografted kidneys with rejection exhibited acute rejection; although the authors stated that their results also applied to allografts with CR, they did not systematically examine that idea and no data were presented. The new expression of the collagen IV α3 and laminin β2 chains at the proximal TBM observed by Abrass *et al.*[13] might thus very well be related to tubulitis during acute rejection. In our study, we observed sporadic new expression of laminin β2 at the TBM in a portion of the biopsy samples in each study group, especially in areas of tubular damage. Staining for this molecule did not differentiate CR from chronic CsAT. We observed increased expression of the collagen IV α3 chain in the CR, CAN, and chronic CsAT groups but no abnormal distribution pattern. We observed a small nonsignificant increase in collagen I staining for the chronic CsAT group, compared with normal control values. It is possible that a small increase in collagen I expression by the patients with chronic CsAT, which was below our detection limit, could have resulted in the expression pattern observed in the study by Abrass *et al.*[13]

In conclusion, we observed a more pronounced fibrotic response in the tubulointerstitium, involving collagen I and III, in allografts with CR, compared with those with chronic CsAT. Early increases in cortical interstitial deposition of collagen I were more specific for CR. Staining for the molecules collagen IV α3 and laminin β2 was not useful for the differentiation of CR from chronic CsAT.

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Chapter 6

Differentiation between chronic rejection and chronic cyclosporine toxicity by analysis of renal cortical mRNA

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Abstract

Background: In kidney transplantation, chronic allograft nephropathy (CAN) is the major cause of graft loss. Causes of CAN include chronic rejection and chronic cyclosporine A (CsA) nephrotoxicity. It is necessary to differentiate between these two entities in order to apply the appropriate therapeutic regimen for the individual patient, but this is hampered by the lack of discriminating functional and morphologic parameters. We investigated whether renal cortical mRNA levels for several matrix proteins can serve as discriminating parameters.

Methods: Patients with chronic rejection (N = 19) and chronic CsA toxicity (N = 17) were selected by clinical and histologic criteria. Protocol biopsies without histologic abnormalities, taken at 6 months after transplantation from patients receiving CsA, were used as controls (N = 6). Total RNA was extracted from the renal biopsy tissue, and mRNA levels of transforming growth factor-β (TGF-β) and the extracellular matrix (ECM) molecules collagen Iα1, IIIα1, IVα3, decorin, fibronectin, and laminin β2 were measured by real-time polymerase chain reaction (PCR).

Results: In both patient groups, the mean collagen IVα3 and fibronectin mRNA levels were significantly elevated compared to those in controls, whereas only in CsA toxicity were the laminin β2 and TGF-β mRNA levels significantly increased. The increase of laminin β2 and TGF-β mRNA levels was significantly higher in the CsA toxicity group than in the chronic rejection group ($P < 0.001$ and $P = 0.004$, respectively). Receiver-operating characteristic (ROC) curve analysis showed that with a 15.6-fold increase in laminin β2 mRNA expression as cut-off point, the presence of CsA toxicity could be predicted with an 87% sensitivity and an 88% specificity.

Conclusion: Renal laminin β2 and TGF-β mRNA levels can be used to differentiate between chronic rejection and chronic CsA toxicity in renal transplants. The method of mRNA quantification might be applicable as an additional diagnostic tool in clinical practice.

Introduction

Over the past decade, renal transplantation has become a very successful treatment modality for end-stage renal disease (ESRD). Due to improvement of immunosuppressive therapy, acute rejection episodes can be treated effectively, and the prevalence of early graft loss has diminished significantly.[1] Long-term graft loss, however, currently forms a major problem in renal transplantation.[1-3]

The term chronic allograft nephropathy (CAN) refers to the pathologic changes, including interstitial fibrosis, tubular atrophy, and fibrous intimal thickening, which are found in chronically dysfunctioning kidney transplants.[2] Several risk factors for CAN have been recognized, such as the number and the severity of acute rejection episodes,[4] ongoing chronic rejection, and excessive exposure to calcineurin inhibitors such as cyclosporine A (CsA).[2,5] Therefore, the lesions that occur in biopsies of patients with CAN may result from either one or a combination of these factors. Paradoxically, changes induced by chronic rejection are clinically and histopathologically hard to distinguish from those caused by the nephrotoxic effects of chronic exposure to CsA, meant to prevent chronic rejection. This makes it difficult to determine the optimal dose of the immunosuppressive regimen, in which the beneficial and nephrotoxic effects of CsA are balanced.[6]

Some changes observed in routine light microscopy may help reveal the cause of chronic renal allograft dysfunction. These include peripheral nodular arteriolar hyalinosis, suggestive of chronic CsA toxicity, and transplant vasculopathy (intimal fibrosis, disruption of the lamina elastica in the presence of inflammation), suggestive of chronic rejection.[7-8] However, these lesions are not decisively present in all patients with either of the diagnoses. Furthermore, due to the fact that peripheral nodular arteriolar hyalinosis and transplant vasculopathy appear focally in the tissue, sampling errors may obscure their presence.

In this study, we describe how differentiation between chronic rejection and chronic CsA toxicity may be improved with the aid of molecular techniques, based on the results of a quantitative analysis of the renal cortical mRNA levels of several extracellular matrix (ECM) components and the ECM-regulating molecule transforming growth factor-β (TGF-β) in two groups of patients suffering from either disease entity.

We focused on several molecules that make up the interstitial compartment of the kidney and are known to accumulate in renal fibrosis, including collagens I and III, and fibronectin, together with the ECM-regulating molecule TGF-β and its potential inhibitor decorin. In recent publications, attention has been drawn to expression of collagen IVα3 and laminin β2 in the discrimination between chronic rejection and CsA toxicity.[9] These molecules are also the subject of the current study.

Methods

Patient populations

We reviewed all kidney transplant biopsies performed in our center over the past 20 years, taken because of renal function loss beyond 1 year after transplantation. We selected two groups of patients: the chronic rejection group and the chronic CsA toxicity group.

The chronic rejection group ($N = 19$), consisted of patients who received either prednisone and azathioprine ($N = 6$), or prednisone and CsA ($N = 13$). Of the 13 patients using CsA, seven received the Sandimmune formulation and six received the Neoral formulation. These patients, with an initially well-functioning kidney transplant, developed a progressive decline in renal function. A biopsy was taken 4.8 ± 3.8 years after transplantation, in which transplant vasculopathy (intimal fibrosis, intimal inflammation, and disruption of the lamina elastica), transplant glomerulopathy [characterized by double contours of the glomerular basement membrane (GBM)] or both were present as a histopathologic indication of chronic rejection, as defined by the Banff 97 classification.[7] Biopsies with peripheral nodular arteriolar hyalinosis, a lesion suggestive of CsA toxicity, and biopsies with signs of *de novo* or recurrent native disease were excluded. Patients suffering from diabetes and patients with graft arterial stenosis were excluded.

The chronic CsA toxicity group ($N = 17$), consisted of patients with an initially well-functioning kidney transplant, who developed a progressive decline in renal function only after a switch was made from Sandimmune to Neoral; CsA formulations with a relatively low and a relatively high bioavailability, respectively. Before the switch, immunosuppression was aimed at CsA 24-hour trough levels of 100 µg/L by administration of Sandimmune once daily. After the switch, Neoral was administered twice daily, aiming at a 12-hour trough level of 150 µg/L. Thereby, the mean daily CsA dose was increased from 3.2 mg/kg to 3.5 mg/kg.[10] Patients who developed a significant and progressive decrease in renal function after this switch, in the absence of other features that might explain the decline in renal function, were included. Renal biopsies, taken 7.1 ± 3.4 years after transplantation and 2.5 ± 1.2 years after the switch from Sandimmune to Neoral, showed peripheral nodular arteriolar hyalinosis in 16 of the 17 patients, histopathologically supporting the functional selection. Biopsies with histologic features suggestive of chronic rejection or *de novo* or recurrent glomerulonephritis were excluded. Patients suffering from diabetes and patients with graft arterial stenosis were excluded.

C4d staining was performed on all biopsy samples. None of the patients in the CsA toxicity group showed C4d depositions in their peritubular capillaries, while 26% of the patients in the chronic rejection showed diffuse C4d depositions in the peritubular capillaries.

Control group

As controls ($N = 6$), we used protocol transplant biopsies taken from patients at 6 months after transplantation with stable graft function at the time of biopsy. Apart from some cases showing signs of minor nonspecific age-related alterations, none of the biopsies showed any signs of rejection or drug toxicity. The glomeruli (at least ten present in the sections for evaluation) did not show any abnormalities. Patients in this control group all used CsA as immunosuppressive medication.

Clinical data

Clinical data included gender, patient age at time of biopsy, donor age, transplant-origin (cadaveric or living donor), number of acute rejection episodes, delayed graft function, time between transplantation and biopsy, time between switch and biopsy, mean arterial pressure (MAP), number of antihypertensive drugs used, use of angiotensin-converting enzyme (ACE) inhibitors, lowest serum creatinine, serum creatinine at biopsy, proteinuria at time of biopsy, and CsA trough levels. We estimated the best endogenous creatinine clearance and creatinine clearance at biopsy using the Cockcroft-Gault equation.[11] The loss of renal function was defined as the best Cockcroft clearance minus the Cockcroft clearance at the time of biopsy.

mRNA isolation and cDNA synthesis

Four micrometer cryostat sections of each biopsy were cut, air dried, and stored at 20°C until use for immunohistochemistry. One section was evaluated to localize the cortex, which was thereafter excised from the biopsy. RNA was subsequently extracted, as described previously.[12] In brief, the tissue was lysed by rigorous mixing after suspension in 500 µL TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA). After adding 100 µL chloroform, the solution was centrifuged at 15,000*g* for 15 minutes. The RNA was precipitated by addition of 5 µg of glycogen and 250 µL isopropanol. cDNA synthesis was performed using an reverse transcription (RT) kit (Omniscript Reverse Transcriptase) (Qiagen GmbH, Westburg B.V., The Netherlands).

Real-time polymerase chain reaction (PCR)

For several ECM molecules and TGF-β1, forward and reverse primers (Life Technologies BRL and Biosource International, Nivelles, Belgium) and TaqMan probes (Biosource International) were designed, using Primer Express® 1.5 software (PE Applied Biosystems, Foster City, CA, USA). To prevent amplification of genomic DNA, primers or probes were chosen spanning an exon-intron junction. Primers were located near the 3' end of the mRNA. The 5' ends of the Taqman probes were 6-carboxy-4,7,2',7'-tetrachloro-fluorescein (TET)-labeled, except those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen IVα3, and decorin, which were 6-carboxy fluorescein (FAM)-labeled. The quencher dye at the 3' side of the probe was 6-carboxy-tetramethyl-rhodamine (TAMRA). The sequences of the primers and of the TaqMan probes are shown in Table 1.
Real-time PCR was performed using the ABI Prism™ 7700 sequence detector and software (PE Applied Biosystems).[13] Amplification cycles were 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds and at 60°C for 60 seconds. Kinetics of the reactions were determined using a standard curve. We used the ratio of the levels of the investigated molecule and GAPDH, a constitutively expressed gene, to correct for the amount of tissue used for RNA extraction and the efficiency of cDNA synthesis. To confirm the suitability of GAPDH as a housekeeping gene, we tested the correlation between the expression of two additional housekeeping genes [*i.e.*, hypoxanthine phosphoribosyl transferase 1 (HPRT1) and β_2-microglobulin (β_2m)], and that of GAPDH in all samples.

Immunofluorescence for C4d

Immunofluorescence staining for C4d was performed on untreated slides using standard procedures as described before.[14-15] As the primary antibody, mouse anti-C4d antibody (Quidel, San Diego, CA, USA), diluted to 2 ng/mL in phosphate-buffered saline (PBS) and supplemented with 1% bovine serum albumin (BSA), was used. The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO, USA), diluted 1:200 in BSA/PBS. The staining was evaluated independently by two of the authors (K.K. and M.E.), blinded for the diagnosis of the samples. C4d staining was observed in the peritubular capillaries (PTC) in a circumferential pattern. Sporadically, mesangial and GBM areas of the glomerulus stained positive. In accordance with scoring methods described in the literature on C4d deposition in renal allografts,[16-17] biopsies were scored C4d-positive when more than 25% of the PTC showed an intense and circumferential staining as depicted in Figure 1. In most cases of diffuse positive samples all PTC were affected. In the few cases of discordant scoring, decision was reached by consensus.

Table 1: Primer and probe sequences

Molecule	Forward primer	Reverse primer	TaqMan™ probe
GAPDH	TGG TCA CCA GGG CTG CTT	AGC TTC CCG TTC TCA GCC TT	5'-FAM-TCA ACT ACA TGG TTT ACA TGT TCC AAT ATG ATT CCA CCA A-TAMRA-3'
β_2m	TGC CGT GTG AAC CAT GTG A	CCA AAT GCG GCA TCT TCA A	5'-TET-TGA TGC TGC TTA CAT GTC TCG ATC CCA CT-TAMRA-3'
HPRT1	TGA CAC TGG CAA AAC AAT GCA	GGT CCT TTT CAC CAG CAA GCT	5'-TET-CTT GAC CAT CTT TGG ATT ATA CTG CCT GAC CA-TAMRA-3'
Collagen Iα1	CCT CAA GGG CTC CAA CGA G	TCA ATC ACT GTC TTG CCC CA	5'-TET-ATG CCT GCA CGA GTC ACA CCG GA-TAMRA-3'
Collagen IIIα1	GAG GAT GGT TGC ACG AAA CA	TGT CAT AGG GTG CAA TAT CTA CAA TAG G	5'-TET-TGA ATA TCG AAC ACG CAA GGC TGT GAG ACT-TAMRA-3'
Collagen IVα3	AAG CCC ACC ACA TGA TTC TGA	GCA GTT GTA GCC AGC CGT ACT	5'-FAM-TCC AAG CAC ACT CCG CAG GCA GT-TAMRA-3'
Decorin	ACA TCC GCA TTG CTG ATA CCA	AGT CCT TTG AGG CTA GCT GCA TC	5'-FAM-TCA CCA GCA TTC CTC AAG GTC TTC CTC C-TAMRA-3'
Fibronectin	GGA GAA TTC AAG TGT GAC CCT CA	TGC CAC TGT TCT CCT ACG TGG	5'-TET-AGG CAA CGT GTT ACG ATG ATG GGA AGA CAT-TAMRA-'3
Laminin β2	GGA TGA GGC TCG GGA CCT	CCC GTC CAA CTG GGC TG	5'-TET-AGG AAT TGG AAG GCA CCT ATG AGG AAA ATG A-TAMRA-3'
TGF-β1	CCC AGC ATC TGC AAA GCT C	GTC AAT GTA CAG CTG CCG CA	5'-TET-ACA CCA ACT ATT GCT TCA GCT CCA CGG A-TAMRA-3'

Abbreviations are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; β_2m, β_2-microglobulin; HPRT1, hypoxanthine phosphoribosyl-transferase 1; TET, 6-carboxy-4,7,2',7'-tetrachloro-fluorescein; FAM, 6-carboxy fluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine.

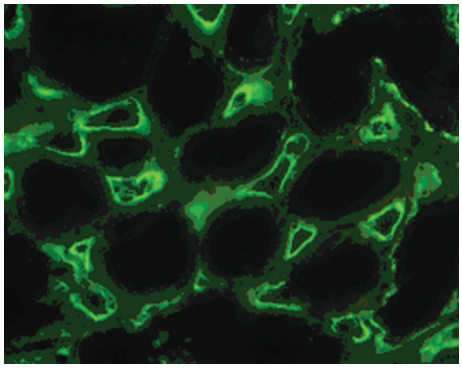

Figure 1: Immunofluorescence staining for C4d. In some chronic rejection samples diffuse circumferential staining of peritubular capillaries for C4d was observed (original magnification x 400).

Immunohistochemistry

For evaluation of protein expression in the tissue, immunohistochemistry was performed for laminin β2 and TGF-β. Four micrometer frozen sections were thawed, air dried, and incubated for 1 hour with mouse monoclonal anti-laminin ß2 antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) diluted 1:16 in BSA/PBS, or rabbit polyclonal anti-TGF-β (Dako, Glostrup, Denmark), diluted 1:400 in BSA/PBS. The anti-TGF-β antibodies stain both the active and latent form of TGF-β. The slides were washed in PBS, and subsequently incubated with horseradish peroxidase (HRP)-conjugated antimouse Envision and HRP-conjugated antirabbit Envision, respectively (Dako). After 45 minutes of incubation, the slides were washed in PBS and the staining was developed with diaminobenzidine (DAB). The color was enhanced by incubating the slides in 0.5% CuSO4 solution for 5 minutes. After counterstaining with hematoxylin, the slides were dehydrated and mounted. For each staining, all biopsy samples were stained in one session.

Digital image analysis

To quantify the amount of staining for TGF-β and laminin β2, images of the cortical part of the biopsies were taken at a 200 magnification using a Zeiss microscope equipped with a Sony DXC-950P 3 CCD color camera (Sony Corporation, Tokyo, Japan) and further

analyzed using KS-400 image analysis software (version 3.0 for Windows) (Carl Zeiss Vision GmbH, Oberkochen, Germany). The cortical area stained was defined as the amount of staining within the color spectrum specific for the enhanced DAB staining, and above a fixed intensity threshold, as described previously.[18] Recording and analysis of the images were performed with fixed settings.

Statistical analysis

Statistical analysis was performed using SPSS 10.0.7 for Windows software. We used log transformed (^{10}log) mRNA levels for analysis. A one-way analysis of variance (ANOVA) with a Bonferroni post hoc correction was used for comparison of differences between groups. Using a receiver-operating characteristic (ROC) curve, we determined the cut-off point of mRNA levels with the best combination of sensitivity and specificity that predicted the presence of CsA toxicity. Correlations between the mRNA data and the clinical characteristics of the patient groups were calculated using Pearson's correlation test. $P < 0.05$ was considered statistically significant.

Results

Patient data

The clinical characteristics of the patients and the controls are listed in Table 2. Renal function at time of biopsy, donor age, patient age, number of acute rejection episodes, delayed graft function, the time interval between transplantation and biopsy, MAP, number of antihypertensive drugs used, and use of ACE inhibitors did not differ significantly between patient groups. There was significantly greater loss of renal function in the chronic rejection group than in the chronic CsA toxicity group (38 ± 16 mL/min and 21 ± 13 mL/min, respectively) ($P < 0.01$). There were significantly more patients with a living-donor kidney transplant in the CsA toxicity group, compared with the chronic rejection group (35% and 5%, respectively) ($P = 0.02$).

Table 2: Clinical characteristics of the patients and the control group

Group	Chronic rejection	Chronic CsA toxicity	Controls
Number	19	17	6
Gender *female*	10 (53%)	4 (24%)	2 (33%)
Number of patients treated with cyclosporine A	13 (68%)	17 (100%)	6 (100%)
Patient age *years* ± *SD*	44 ± 14	49 ± 13	45 ± 6
Donor age *years* ± *SD*	35 ± 16	44 ± 10	47 ± 17
Living-donor kidney transplants *%*	5	35[c]	
Number of acute rejection episodes	0.8 ± 1.0	0.5 ± 0.6	
Delayed graft function *%*	21	18	
Time between transplantation and biopsy *years* ± *SD*	4.8 ± 3.8	7.1 ± 3.4	
Time between switch and biopsy *years* ± *SD*		2.5 ± 1.2	
Mean arterial pressure	108 ± 10	106 ± 8	
Number of antihypertensive drugs used (0/1/2/≥3)	2/2/8/7	2/5/5/5	
Use of ACE-inhibitors *%*	21	29	
Best serum creatinine level µ*mol/L* ± *SD*	113 ± 27	124 ± 25	
Serum creatinine at time of biopsy µ*mol/L* ± *SD*	238 ± 83	201 ± 45	118 ± 25
Best creatinine clearance *mL/min* ± *SD*[a]	74 ± 22[c]	61 ± 11	
Creatinine clearance at time of biopsy *mL/min* ± *SD*[a]	36 ± 14	40 ± 15	68 ± 14
Loss of renal function µ*mol/L* ± *SD*[b]	38 ± 16[d]	21 ± 13	
Proteinuria at time of biopsy *g/24hours* ± *SD*	2.9 ± 2.6	1.3 ± 1.5	
Cyclosporine A trough levels at time of biopsy µ*g/L* ± *SD*	110 ± 31	114 ± 30	

[a] Creatinine clearance was estimated by the Cockcroft-Gault equation.

[b] Loss of renal function was defined as the difference between the best creatinine clearance and the creatinine clearance at the time of biopsy.

[c] $P < 0.05$; [d] $P < 0.01$.

Abbreviations are: ACE angiotensin-converting enzyme.

Cortical mRNA levels

GAPDH mRNA levels did not differ between groups (data not shown). Within the chronic rejection group, mean GAPDH expression did not significantly differ between CsA-using and non-CsA-using patients. There was a significant correlation between GAPDH mRNA and mRNA of the two other housekeeping molecules measured ($r = 0.74$ and $r = 0.92$

for β_2m and HPRT1, respectively) ($P < 0.001$). Additionally, we tested comparisons between the chronic rejection and the CsA toxicity group for all transcripts using β_2m as the housekeeping molecule. This yielded the same results as when GAPDH was used. These findings support the suitability of GAPDH as a housekeeping molecule in the experiments.

Table 3: Log transformed mRNA levels[a].

	Total chronic rejection (Ia)	Chronic rejection using CsA (Ib) [b]	Chronic CsA toxicity (II)	Controls (III)
Collagen Iα1	0.3 ± 0.2	0.2 ± 0.3	0.2 ± 0.3	0.8 ± 0.2
Collagen IIIα1	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
Collagen IVα3	0.1 ± 0.2[c]	0.1 ± 0.2[c]	0.5 ± 0.1[c]	-1.9 ± 0.3
Decorin	0.0 ± 0.2	-0.2 ± 0.2	-0.1 ± 0.2	-0.4 ± 0.2
Fibronectin	-0.1 ± 0.2[c]	-0.3 ± 0.2[c]	-0.1 ± 0.2[c]	-1.6 ± 0.2
Laminin β2	0.6 ± 0.1[e]	0.5 ± 0.2[e]	1.1 ± 0.1[c]	-0.1 ± 0.3
TGF-β	0.8 ± 0.1[d]	0.8 ± 0.2[d]	1.3 ± 0.1[c]	0.5 ± 0.2
Ratio TGF-β / decorin	0.8 ± 0.2[d]	0.8 ± 0.2[d]	1.4 ± 0.1	0.9 ± 0.2

[a] All values are mean ± SEM; [b] Mean log transformed mRNA levels of the chronic rejection group after omission of patients using azathioprine as immunosuppression; [c] $P < 0.01$, *vs.* III.; [d] $P < 0.05$, *vs.* II, [e] $P < 0.01$, *vs.* II; Abbreviations are: mRNA, messenger ribonucleine acid; CsA, cyclosporine A; TGF-β, transforming growth factor-β.

The mean log transformed mRNA levels of collagen Iα1, collagen IIIα1, collagen IVα3, decorin, fibronectin, laminin β2, and TGF-β are shown in Table 3 and depicted in Figure 2. The mRNA levels of collagen IVα3 and fibronectin were higher in both patient groups compared to controls. The mRNA levels of laminin β2 and TGF-β were higher in the CsA toxicity groups compared to controls (Table 3) (Fig. 2F and H). The renal mRNA levels of collagen Iα1, IIIα1, and IVα3, decorin, and fibronectin were not significantly different between patients with chronic rejection and patients with CsA toxicity (Table 3) (Fig. 2A to E). The renal mRNA levels of laminin β2, TGF-β, and the ratio of TGF-β to decorin were significantly higher in patients with CsA toxicity than in patients with chronic rejection (Table 3) (Fig. 2F and G). After omitting patients who did not use CsA from the chronic rejection group, comparative analyses between groups yielded comparable results (Table 3). In addition, there were no differences in mRNA expression of all molecules analyzed between chronic rejection patients who used CsA and those who did not use CsA as immunosuppression (collagen Iα1, $P = 0.44$; collagen IIIα1, $P = 0.51$; collagen IVα3, $P = 0.57$; decorin, $P = 0.07$; fibronectin, $P = 0.10$; laminin β2, $P = 0.52$; TGF-β, $P = 0.53$). There were no significant differences between mRNA expression levels in cadaveric transplants and living-donor transplants within each patient group.

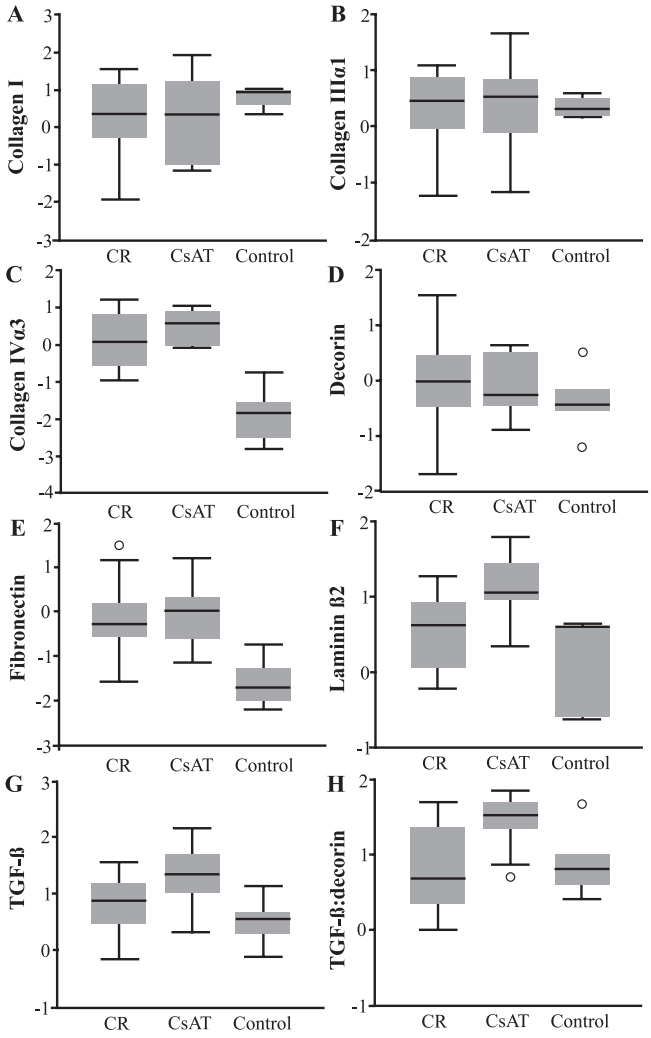

Figure 2: Box and whisker plots of the log transformed mRNA levels. The boxes contain 50% of the values. The upper and lower border indicate the 25th and the 75th percentile, respectively. The upper and lower whisker indicate the highest and lowest value, respectively. The black line in the box indicates the median and the (◦) indicates an outlier. (A) Collagen Iα1. (B) Collagen IIIα1. (C) Collagen IVα3. [$P < 0.001$ chronic rejection (CR) and cyclosporine A (CsA) toxicity (CsAT) *vs.* controls]. (D) Decorin. (E) Fibronectin ($P < 0.001$ chronic rejection and CsA toxicity *vs.* controls. (F) Laminin β2 ($P < 0.001$ CsA toxicity *vs.* controls ($P = 0.002$ chronic rejection *vs.* CsA toxicity). (G) Transforming growth factor-β (TGF-β) ($P = 0.004$ CsA toxicity *vs.* controls) ($P = 0.020$ chronic rejection *vs.* CsA toxicity). (H) TGF-β/decorin ratio ($P = 0.014$ chronic rejection *vs.* CsA toxicity).

Immunohistochemistry

We performed immunohistochemical stainings to evaluate the tissue expression of laminin β2 and TGF-β at the protein level (Figure 3). In biopsies of controls, laminin β2 staining was observed in the GBM and in cortical vessels (Figure 3A). In chronic rejection as well as in CsA toxicity, sporadic expression of laminin β2 was seen in the tubular basement membrane (Figure 3B and C). TGF-β staining was sporadically observed in glomeruli and tubuli of controls (Figure 3D). In sections of biopsies from patients suffering from chronic rejection or CsA toxicity, some tubuli showed very intense staining for TGF-β (Figure 3E and 3F). There was no relation between this sporadic intensive staining and mRNA expression levels or clinical parameters.

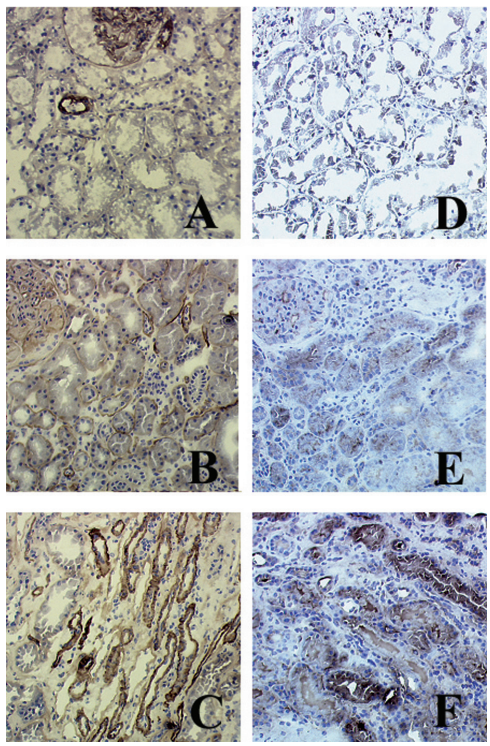

Figure 3: Immunohistochemical stainings for laminin β2 (A to C) and transforming growth factor-β (TGF-β) (D to F). In control tissue laminin β2 staining was observed in the glomerular basement membrane and cortical vessels (**A**) In chronic rejection (**B**) and cyclosporine A (CsA) toxicity (**C**) expression of laminin β2 was observed in the tubular basement membrane. TGF-β staining was sporadically observed in glomeruli and tubuli of controls (**D**) In chronic rejection (**E**) and CsA toxicity (**F**), some tubuli showed a very intense staining for TGF-β.

In both patient groups, the amount of laminin β2 staining was increased compared to controls. The mean cortical area percentage was slightly higher in the CsA toxicity group than in the chronic rejection group (5.7 ± 1.5 and 5.3 ± 1.0, mean ± SEM) (Figure 4A). The differences between groups were not significant. The amount of TGF-β staining was higher in patient groups than in controls. The mean cortical area percentage was higher in the CsA toxicity group than in the chronic rejection group (15.8 ± 3.6 and 13.4 ± 2.2, mean ± SEM) (Figure 4B), though this difference did not reach statistical significance.

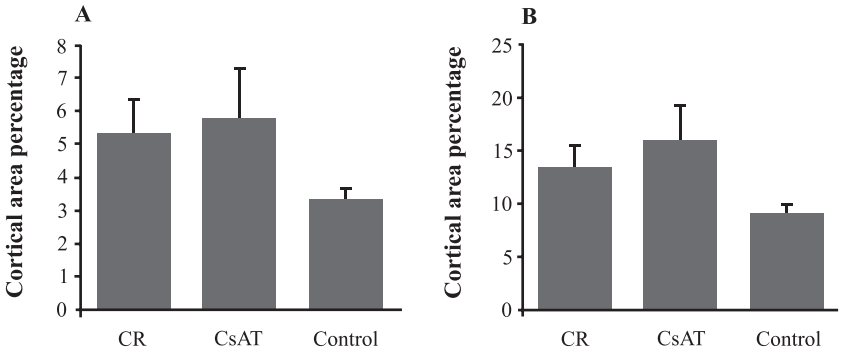

Figure 4: Immunohistochemical staining for laminin β2 and transforming growth factor-β (TGF-β). Bars represent mean cortical area percentage ± SEM. (**A**) The amount of laminin β2 staining is slightly higher in the cyclosporine A toxicity (CsAT) group compared to the chronic rejection (CR) group, but the difference between groups does not reach statistical significance. (**B**) TGF-β staining is increased in the CsA toxicity group compared to the chronic rejection group. There are no significant differences between groups.

ROC curve analysis

ROC curve analysis (Figure 5) showed that a 15.6-fold increase of laminin β2 mRNA levels compared to controls indicates the presence of CsA toxicity with an 87% sensitivity and an 88% specificity. The area under the curve was 0.90 ($P < 0.001$). Similarly, a ninefold increase of TGF-β mRNA levels predicts the presence of chronic CsA toxicity with a 60% sensitivity and an 88% specificity (area under the curve 0.76) ($P < 0.05$).

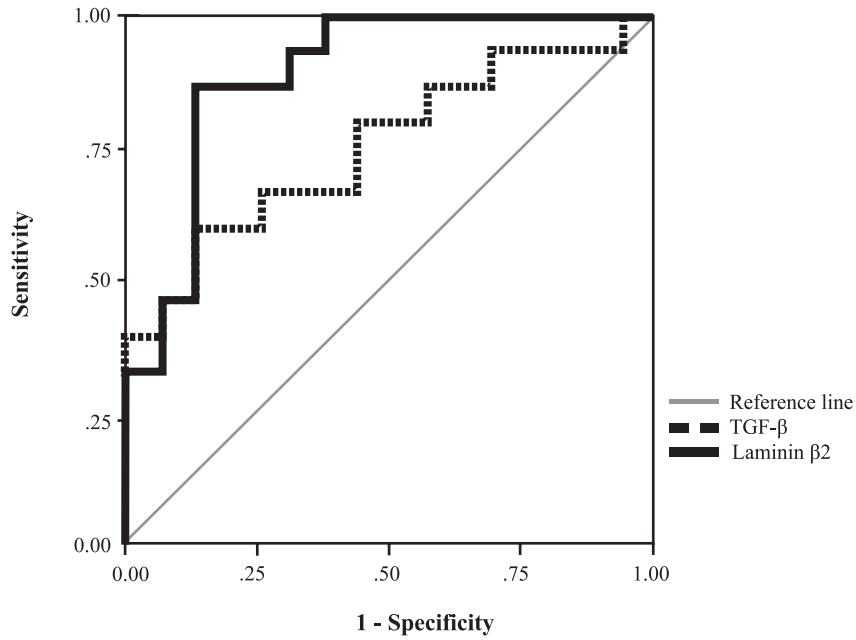

Figure 5: Receiver-operating characteristic (ROC) curve of laminin β2 and transforming growth factor-β (TGF-β). The fraction of true positive results (sensitivity) and false-positive results (1-specificity) for laminin β2 (solid line) and TGF-β (dashed line) mRNA levels. The area under the curve indicates the accuracy of the test: 0.5 is the value expected by chance (diagonal line), 1.0 represents the ideal predictor. The area under the curves for laminin β2 and TGF-β are 0.896 and 0.758, respectively. Using a 15.6-fold increase in laminin β2 mRNA levels to those of normal controls as cut-off point, the sensitivity is 87% and the specificity 88% for the prediction of the presence of chronic cyclosporine A toxicity.

Correlations

No correlations were found between mRNA levels and age, donor age, number of acute rejection episodes, delayed graft function, time between transplantation and biopsy, the time between switch and biopsy, loss of renal function, MAP, and use of ACE inhibitors. Only in the chronic rejection group, TGF-β mRNA levels and the ratio of TGF-β to decorin mRNA levels correlated significantly with laminin β2 mRNA levels ($r = 0.56$ and $r = 0.68$, respectively) ($P < 0.05$).

Discussion

This study was designed to enhance the discrimination between chronic rejection and chronic CsA toxicity as a cause of CAN. With current clinical and histologic parameters this distinction is difficult to make because of the similarities in clinical presentation and aspecificity of the lesions in needle biopsies of patients with CAN. We show that quantification of mRNA levels of laminin β2 and TGF-β can be used to distinguish chronic rejection from CsA toxicity, which will help in fine-tuning the immunosuppressive regimen. In this way, the beneficial effects of CsA are not abrogated by its nephrotoxic side effects.[3]

In our center, we had the opportunity to define on functional criteria a patient group that suffered from chronic CsA toxicity, comprising patients who developed a progressive decline in renal function after a change in immunosuppressive medication was made leading to a higher CsA exposure.[10] The appropriateness of these selection criteria was supported by histopathologic findings: peripheral nodular arteriolar hyalinosis, regarded as a finding suggestive of CsA toxicity, was present in 94% of these patients, and C4d deposition in PTCs, a feature frequently seen in chronic rejection,[19] was absent in all patients of the CsA toxicity group. An additional selection criterion for the CsA toxicity group and a further proof of CsA toxicity might have been the recovery of renal function after stopping or reducing CsA administration. Unfortunately, these data were not available. In the chronic rejection group, 13 out of 19 patients also received CsA. However, since the CsA formulation in the majority of these patients had a relatively low bioavailability and administration was applied only once-daily, this group is less likely to have suffered from CsA toxicity. In addition, patients in this group had transplant vasculopathy or glomerulopathy, histologic features suggestive of chronic rejection, but they did not show histologic features consistent with CsA toxicity. Finally, although there was some heterogeneity in immunosuppressive medication in the chronic rejection group, no differences were found in mRNA data between patients using CsA and those who did not use CsA as immunosuppression, and analysis with only the group of chronic rejection patients using CsA as immunosuppression yielded the same results as the chronic rejection group as a whole. A striking difference in patient characteristics was that 35% of the CsA toxicity group, but only 5% of the transplants in the chronic rejection group were living donor transplants. This might support the opinion that injuries inflicted to cadaveric allografts before or during transplantation elicit a predisposition for the development of chronic rejection, as has been suggested before.[2,20]

The ECM is a meshwork of proteins, in which remodeling continuously takes place by means of protein synthesis and degradation. Accumulation of ECM proteins reflects an

imbalance of this dynamic process, resulting from an increase in protein synthesis, a decrease in protein degradation, or a combination of both. The synthesis of ECM components is enhanced by several profibrotic cytokines, including TGF-β. During chronic renal allograft dysfunction, this cytokine has been shown to be up-regulated at the mRNA and protein level in the grafts, sera, and peripheral blood mononuclear cells of patients taking CsA-based immunosuppression.[21-22] TGF-β mRNA levels are up-regulated in cultured murine proximal tubular epithelial cells and fibroblasts after exposure to CsA.[23] Moreover, in rats receiving CsA, administration of anti-TGFβ-1 antibodies reduces the extent of histologic damage reminiscent of CsA toxicity.[24] Decorin, a low-molecular-weight proteoglycan, can bind and inactivate TGF-β, thereby preventing its prosclerotic action.[25] The TGF-β/decorin mRNA ratio may therefore be a better indicator of TGF-β activity than TGF-β mRNA levels alone. We found a significant increase of TGF-β mRNA levels and of the TGF-β/decorin ratio in CsA toxicity, supporting the notion that CsA has a stimulatory effect on TGF-β expression.

Laminin β2 is a normal component of the renal vasculature and the GBM.[26] We observed an increase in laminin β2 mRNA expression in the CsA toxicity group compared to controls, the expression in the CsA toxicity group being also significantly higher than in the chronic rejection group. Laminin β2 mRNA expression was also higher in the CsA toxicity group when comparing it to the chronic rejection group with only those patients using CsA included. Only sparse information about factors stimulating laminin β2 expression is available. The results of our study suggest a direct or indirect stimulatory effect of CsA on laminin β2 expression, yet the underlying mechanism is unclear.

We performed immunohistochemistry combined with digital image analysis for laminin β2 and TGF-β to evaluate the expression of these molecules at the protein level. The pattern seen at the protein level resembled that at the mRNA level (*i.e.*, there was a tendency toward a higher expression of laminin β2 and TGF-β in the CsA toxicity group than in the chronic rejection group). However, differences in protein staining between groups did not reach statistical significance. Furthermore, a correlation between mRNA expression levels and protein deposition was absent. The observation that the extent of protein accumulation does not strictly coincide with mRNA levels has been described before.[27] Additionally, in vivo accumulation of protein is not only determined by synthesis, but also by degradation of ECM products. Although we did not evaluate the mechanism of laminin β2 degradation, the notion that laminin β2 mRNA levels were increased in the CsA toxicity group compared to the chronic rejection group, while protein levels were not significantly different, might suggest that there is an increased degradation of laminin β2 in CsA toxicity. This might be due to the microvascular damage exerted by CsA.[8]

Collagen Iα1 and collagen IIIα1 are components of the renal interstitium that are normally present in relatively small amounts.[28] Accumulation of these molecules has been reported in a variety of chronic human kidney diseases[29] and CAN.[30-31] There was no difference between the chronic rejection group and the CsA toxicity group in collagens I and III mRNA levels. We observed no differences in the mRNA levels between the patient groups and the control group. This might be explained by the possibility that the accumulation of collagens I and III in CAN is a result of an early increase in collagen synthesis that might have taken place before the overt damage seen in the tissues. Furthermore, it might be that the accumulation of collagens I and III in patients with CAN is due to an impaired degradation of these proteins, as has been suggested before.[32]

Collagen IVα3 is a component of both the GBM and the distal tubular basement membrane (TBM).[26] In a study by Abrass *et al.,*[9] de novo expression of collagen IVα3 protein was reported in the proximal TBM in chronic rejection, but not in CsA toxicity. In our study, we did not observe significant differences in collagen IVα3 mRNA expression levels between the two groups. Since we used total cortical tissue for mRNA analysis, it is possible that subtle differences of collagen IVα3 mRNA expression in the proximal tubulus epithelium between patient groups remained undetected. When we focused only on the chronic rejection group, the collagen IVα3 mRNA expression was higher in the C4d+ chronic rejection group than in the C4d- chronic rejection group (data not shown). C4d recently has gained much interest as a marker of humoral rejection, but there is only sparse information about the relation between C4d and accumulation of ECM.[33] Future studies would be needed to decipher whether the relation found in our study is of pathogenic significance.

In both the chronic rejection and the chronic CsA toxicity group, we observed a significant increase in renal cortical fibronectin mRNA levels in comparison to controls. There were no differences in the mRNA levels of fibronectin between the chronic rejection and the CsA toxicity group. This is in line with previous studies showing an increase in fibronectin mRNA levels in a rat model of CsA toxicity,[34] and in allograft rejection, both at the mRNA and the protein level.[35-36]

One of the advantages of analyzing mRNA profiles may be that alterations in mRNA levels precede the development or aggravation of tissue damage.[37] This holds promise for an earlier recognition of an unfavorable course after kidney transplantation.[38-39] Furthermore, quantitative mRNA analysis can be performed rapidly, and requires only small amounts of renal tissue. In the future, diagnostic approaches using molecular analysis simultaneously with conventional strategies are likely to be implemented in clinical practice.[40] We had the opportunity to compose merely on the basis of functional variables two patient groups that represent the extremes of a spectrum of causes leading to the development of CAN. Therefore, we studied the presence of markers that could discriminate between these two

highly selected patient groups. An obvious prerequisite for implementation in clinical practice is to test the use of these markers in larger nonselected patient groups.

Conclusion

We measured human renal cortical mRNA expression levels of ECM components in two well-defined groups of patients suffering from either chronic rejection or chronic CsA toxicity. In both patient groups, the mean mRNA levels for collagen IVα3 and fibronectin were significantly elevated compared to those in controls, whereas in CsA toxicity the laminin β2 and TGF-β mRNA levels were also significantly increased. Most important, we showed that laminin β2 and TGF-β mRNA levels are significantly higher in patients with CsA toxicity than in patients with chronic rejection, and that measurement of these expression levels may help differentiate chronic CsA toxicity from chronic rejection.

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Chapter 7

Summary and discussion

Summary and discussion

Late transplant dysfunction or loss remains an important problem after kidney allograft transplantation. A gradual but variable rate of decline in allograft function is observed in about 40 to 50% of the patients who receive a kidney allograft transplant at two or more years after transplantation.[1] Various factors, alloantigen-dependent and alloantigen-independent, may play a role in the loss of function. A biopsy of the allografted kidney is often taken after prerenal and postrenal causes of functional decline are excluded to help to design a strategy that prevents further loss of function. In a small minority of cases histologic changes are found that suggest a specific cause of graft dysfunction such as a recurrence of the original kidney disease, *de novo* glomerulopathy or diabetic nephropathy. More frequently less specific chronic and sclerosing signs of tissue damage are observed.
Since 1991 efforts have been made to standardize the interpretation of pathologic findings of renal allograft biopsies in the Banff Working Classification of Renal Allograft Pathology. In this system the descriptive term chronic allograft nephropathy (CAN) was adopted for chronic and sclerosing tissue changes, which include arteriosclerosis, glomerulosclerosis, tubular atrophy, interstitial fibrosis, and tubular atrophy.[2] The severity of these changes is staged by the degree of the interstitial fibrosis and tubular atrophy. The pathogenesis of CAN is multifactorial and comprises factors that are alloantigen-independent and alloantigen-dependent.[3,4] The tissue changes of CAN may represent cumulative and incremental damage to nephrons from time-dependent insults.[5] The immune-mediated alloantigen-dependent injury may result from acute rejection, subclinical acute rejection or chronic rejection. Alloantigen-independent injury may result from ischemia/reperfusion, drug nephrotoxicity, viral infection and primary vascular changes due to hypertension, smoking or dyslipidemia. Also the age of the allografted kidney plays a role and may determine whether tissue damage occurs and how this is repaired. The kidneys from older donors are more susceptible to acute rejection and the ageing kidney seems to have an impaired ability to restore tissue.[6]
Although CAN may represent the final histologic appearance of many different insults to the allografted kidney, it remains important to identify factors that are still initiating tissue damage. Some specific histologic features may indicate ongoing immune-mediated alloantigen-dependent damage. Chronic allograft glomerulopathy (CAG), also known as chronic transplant glomerulopathy (TGP), represents an alloantigen-dependent, probably antibody-mediated, injury at the level of the glomeruli. CAG is found in a small minority (5-15%) of late biopsies with features of CAN.[7] *De novo* arterial intimal fibrosis also supports the diagnosis of chronic alloantigen-dependent damage. However, most of the time it is not clear whether the arteriosclerotic lesions occurred de novo, or alternatively,

were already present at transplantation or induced by cardiovascular risk factors operating in the acceptor.

Newer methods of studying immune-mediated damage in late allograft biopsies include the immunostaining for C4d in the peritubular capillaries (PTCs), and the electron microscopic examination of the peritubular capillary basement membranes. C4d is a split product of the complement factor C4 that covalently binds to tissue and its presence in PTCs has been studied as a possible marker for acute and chronic antibody- mediated rejection. At present definite evidence is still lacking that positive C4d staining of PTCs in a biopsy with CAN indicates ongoing antibody-mediated rejection.[8] It has been recognized that extensive multilayering of peritubular capillary basement membranes at electron microscopy is strongly associated with CAG.[9-11] Its significance as an isolated finding in biopsies with CAN has not been established. More studies are needed to evaluate the importance of these new techniques in the diagnosis of CR.

Drug toxicity may affect the graft, especially of the calcineurin inhibitors (CNI) cyclosporine A (CsA) and tacrolimus, which are used to prevent rejection. An important marker of chronic CNI toxicity is peripheral nodular hyaline degeneration (PNHD) of small arterioles. However, the changes of PNHD may be difficult to separate from the less specific, more commonly observed lesions of arteriolar hyalinosis.[12] Furthermore, as a marker of chronic CNI nephrotoxicity, they seem to be not very sensitive.[12] Although extensively studied, the pathophysiologic characteristics of chronic CNI nephrotoxicity are still far from being completely understood. Calcineurine inhibitors are the most widely prescribed immunosuppressive drugs in organ transplantation and the use of these drugs has allowed significant improvement in the short-term success rate of kidney transplantation. With respect to their nephrotoxic potential, uncertantity still exists how severely they affect long-term graft survival and whether they should be stopped at some point after transplantation.

This thesis describes studies that focus on chronic CsA nephrotoxicity, and on tissue changes that occur in the tubulointerstitial compartment of kidneys with CAN. Several issues were addressed: the contribution of chronic CsA nephrotoxicity to late allograft dysfunction and CAN; the direct tubulotoxicity of CsA; the composition of the renal cortical interstitial matrix; and the measurement of renal cortical mRNA of various proteins involved in extracellular matrix (ECM) deposition.

In **chapter 2** the present knowledge on chronic CsA nephrotoxicity is reviewed. Although extensively studied, the pathophysiology of chronic CsA nephrotoxicity is still far from being completely understood. The recognition of chronic CsA nephrotoxicity in allografted kidneys is hampered by the lack of easily assessable sensitive and specific markers. It has been recognized that more than half of the patients that suffer from late allograft

deterioration and who have CAN in their biopsy benefit from withdrawal of CsA or a reduction of its dose.[13] This strongly suggests that chronic CsA nephrotoxicity has an important contribution to late allograft deterioration and CAN. Several hypotheses attempt to explain the renal structural damage induced by CsA, including ischemia of the graft as a result of functional or structural vascular changes, direct cellular toxicity towards renal cells, and the stimulation of interstitial matrix production through induction of growth factors and cytokines. Possible ways to avoid chronic CNI nephrotoxicity are discussed and it is concluded that in the near future more strategies are likely to be used to prevent loss of allograft function as a result of CNI nephrotoxicity.

Chapter three examines whether CsA is directly toxic for human proximal tubular epithelial cells. The exact pathogenesis of chronic CsA nephrotoxicity is, after 25 years of use of the drug, still largely unknown. Morphological studies reported proximal tubular epithelial cell vacuolization and inclusion bodies early after transplantation during CsA treatment, and animal and human studies have found an increase in the urinary excretion of the proximal brush border enzyme N-acetyl-β-D-glucosaminidase.[14] Moreover the urinary excretion of β_2-microglobulin is enhanced during CsA therapy suggesting proximal tubular cell damage.[15] In both human and animal studies a higher rate of tubular cell apoptosis has been described during CsA exposure.[16] Still it was not clear whether this increased apoptotic activity is the result of a direct toxic effect of CsA or the result of an indirect mechanism such as ischemia. It was hypothesized that a very high supratherapeutic concentration of the drug induces tubular cell necrosis and that lower, more therapeutic concentrations promote apoptosis. The aim of the studies, as described in this chapter, was to examine whether CsA directly induces cell death of cultured proximal tubular epithelial cells (PTEC) by either necrosis or apoptosis. Therefore primary isolates of human PTEC and LLC-PK_1-cells, a pig immortalized cell line of proximal descent, were examined at various points after CsA exposure at concentrations of 0.01-100 μg/ml. Apoptosis was studied by the assessment of annexin binding and propidium iodide uptake, the measurement of cellular DNA content and cell cycle analysis and by the evaluation of nuclear morphology. Cell death was studied by the trypan blue exclusion method. Experiments were also performed under simulated hypoxic conditions by chemical ATP depletion, because of the possibility that *in vivo* PTEC could be ATP depleted when exposed to CsA.

In human PTEC, cell death was observed at CsA concentrations higher than 10 μg/ml; at these concentrations, PTEC died as a result of necrosis and the toxicity of its vehicle Cremophore EL, and not as a result of CsA inducing apoptosis. The addition of cycloheximide to relieve a possible block in the apoptotic process had no effect on human PTEC but resulted in apoptosis of LLC-PK_1. In human PTEC, CsA did not augment cell death induced by chemical ATP depletion.

Earlier studies on CsA cytoxicity in cultured renal tubular cells had reported contradictory results. Only two studies reported loss of viability of cultured human PTEC .[17,18] However, in the first study the cells were deprived of essential nutrients before incubation with CsA and in the second study the cells were of foetal origin. We concluded that the results of our *in vitro* study argued against a direct cytotoxic effect of CsA on adult human proximal tubular epithelial cells *in vivo*.

Chapter 4 describes the results of a long-term open-label prospective randomized study that was initiated in 1983 and compared cyclosporine continuation with conversion to azathioprine three months after transplantation.

It has long been recognized that CsA has many toxic side effects: it raises the blood pressure and serum cholesterol level and may cause nephrotoxicity, all of which contribute to mortality and graft loss in the late post-transplantation period. It was hypothesized that reduction in exposure to the drug after the period with the highest risk of acute rejection would be advantageous. It was hoped that the withdrawal of cyclosporine and the replacement by azathioprine after a certain time post-transplantation would preserve the better short-term results of cyclosporine therapy while avoiding the consequences of long-term exposure to the drug. In 1995 data on 8 years follow-up were published.[19] No significant difference was found in patient and transplant survival. There was an apparent trend over time of an increasing difference in transplant survival in favor of the patients that converted to azathioprine. Benefits were noted on side effects and cardiovascular risk factors.

Chapter 4 reports the 15-year analysis of outcome and provides data on histological abnormalities in the allograft biopsies. One hundred-twenty-eight patients were enrolled. Three months after transplantation they were randomly assigned to continue cyclosporine treatment ($N = 68$) or to convert to azathioprine treatment ($N = 60$). The steroid dose was temporarily increased in the patients who were converted. Patient survival was not different in the two groups. Graft survival tended to be lower (64.7% versus 76.5% at 15 years) in the cyclosporine continuation group ($P = 0.14$), when data were analyzed on an intention-to-treat basis. More allograft biopsies were taken from patients remaining on cyclosporine and prompted a high rate of late conversions to azathioprine or mycophenolate mofetil for suspicion of chronic CsA nephrotoxicity (19%). This could have influenced the results in favor of the CsA continuation arm. The graft survival of the patients that stayed on their assigned treatment was significantly higher in the azathioprine arm, starting at two years' post-transplantation. The glomerular filtration rate was significantly higher in the patients who were converted to azathioprine at all points in time. The relative risk of CAN was significantly higher in the group that continued cyclosporine (relative risk 4.3, [95% CI: 1.4 to 12.9], $P = 0.009$). Conversion to azathioprine reduced the need for blood pressure

and lipid lowering drugs. It was concluded that conversion to a calcineurin inhibitor-free immunosuppressive regimen three months after renal transplantation improved allograft function, reduced the need for cardiovascular risk factor controlling medication and reduced the incidence of CAN.

The results of this long-term study emphasize that withdrawal of CsA after a critical time frame can be advantageous in terms of graft survival. The results are in line with the 3-year data of a study that used sirolimus as the agent to continue along with steroids after withdrawal of CsA at three months. The 3-year results showed a 6% higher graft survival (P = 0.052) and a significantly higher GFR of 12 ml/min;[20] biopsy samples showed significantly lower scores for chronic sclerosing lesions in the patients that were withdrawn from CsA.[21] In this latter study, as well as in our study, cyclosporine treatment was monitored on trough level (C0) measurements. It has been shown that newer ways of therapeutic drug monitoring (TDM) of CsA, such as limited sampling methods or C2 monitoring, leads to a decrease in the incidence of acute rejection. However, the effect of these newer ways of TDM on the incidence of chronic CsA nephrotoxicity is not yet known. It should also be mentioned that the results of our trial and that of the sirolimus continuation trial may not apply to patients with a high risk profile for rejection.

The more prominent changes in biopsies with CAN occur in the tubulointerstitial compartment with increased deposition of ECM. **Chapter 5** examines the composition of the cortical interstitial matrix of patients whose allograft function declined as a result of CR or chronic CsA nephrotoxicity. Extracellular matrix accumulates in the cortical interstitium of dysfunctioning allografts regardless of the etiology, as is seen in chronic diseases of native kidneys.[22] Surprisingly, the ECM composition of deteriorating allografted kidneys has hardly been studied. Defining the ECM molecules that accumulate could enhance our understanding of the pathogenesis of graft dysfunction and could potentially help define the etiology if disease-specific changes are found. In this study we investigated whether the ECM composition differs in allografts with CR or chronic CsA nephrotoxicity. For this purpose, we studied the cortical interstitial ECM composition of kidney allografts with chronic CsA nephrotoxicity, with CR, and with suspected CR, here designated as CAN. The patients with CR used a CNI free immunosuppressive regimen. The patients with suspected CR were on an immunosuppressive regimen that contained CsA but their biopsy findings strongly suggested CR as the main cause of graft decline. The patients with chronic CsA nephrotoxicity developed deteriorating graft function only after a switch to a CsA formulation with a higher bioavailability several years after transplantation.

The expression of the proteins collagen I, III, and IV, collagen IV α3, laminin β2 and α-smooth muscle actin (SMA) was studied in allograft biopsies with chronic CsA nephrotoxicity (N = 17), chronic rejection (N = 12), or CAN (N = 19). Tissue from normal native kidneys

was used as control ($N = 11$). Biopsy samples were studied by routine light microscopy and after immunostaining. The area taken up by the stain was measured digitally. Glomeruli and blood vessels were excluded from the analysis. The mean interstitial fibrosis scores were significantly higher in the CR and CAN group than in the chronic CsA nephrotoxicity group. The cortical tubulointerstitial areas of the CR and CAN group, but not of the chronic CsA nephrotoxicity group, contained more collagen I than normal controls. A difference was already noted in biopsies with mild interstitial fibrosis. The collagens III, IV and IV α3 were increased in all groups. Collagen III accumulated to a greater extent in the CR and CAN group than in the chronic CsA nephrotoxicity group. Receiver-operating characteristic (ROC) curve analysis demonstrated that collagen I staining had the best discriminatory value in differentiating CR from chronic CsA nephrotoxicity, with a sensitivity of 63% and specificity 94% at a cut-off value of 19% cortical interstitial staining. Laminin β2 staining did not differentiate CR from CsA nephrotoxicity and no significant difference was noted with the control group. Increases in SMA staining were observed for all patient groups, compared with the control group, but no differences were observed among the patient groups. We concluded that, during chronic CsA nephrotoxicity, collagen III and IV were preferentially accumulated in the tubulointerstitium. Early increases in the deposition of collagen I, along with collagens III and IV, were more specific for CR. CR seems to elicit a more pronounced fibrotic response than does chronic CsA nephrotoxicity. Our results do not support the conclusion of a report by Abrass et al.,[23] which suggested that a pattern of new expression of laminin β2 and collagen IV α3 at the proximal tubular basement membrane is specific for CR. Our findings suggest that the specific composition of the accumulated tubulointerstitial ECM in biopsies with CAN is influenced by the etiologic factors that induced the ECM deposition.

In **chapter 6** we studied the renal cortical mRNA levels of several extracellular matrix (ECM) components and the ECM-regulating growth factor transforming growth factor β (TGF-β). Patients with CR ($N = 19$) and chronic CsA nephrotoxicity ($N = 17$) were selected by clinical and histological criteria. Protocol biopsies without histologic abnormalities, taken at 6 months after transplantation from patients receiving CsA, were used as controls ($N = 6$). Total RNA was extracted from the renal biopsy tissue, and mRNA levels of TGF-β and the extracellular matrix (ECM) molecules collagen Iα1, IIIα1, IVα3, decorin, fibronectin, and laminin β2 were measured by real-time polymerase chain reaction (PCR). In both patient groups, the mean collagen IVα3 and fibronectin mRNA levels were significantly elevated compared to those in controls, whereas only in CsA toxicity were the laminin β2 and TGF-β mRNA levels significantly increased. The increase of laminin β2 and TGF-β mRNA levels was significantly higher in the CsA toxicity group than in the CR group ($P < 0.001$ and $P = 0.004$, respectively). ROC curve analysis showed that with

a 15.6-fold increase in laminin β2 mRNA expression as cut-off point, the presence of CsA toxicity could be predicted with an 87% sensitivity and an 88% specificity. We concluded that renal laminin β2 and TGF-β mRNA levels are possible new molecular markers to differentiate chronic CsA toxicity from CR in renal transplants.

In chapters 5 and 6 a search was done for new specific markers of either CR or chronic CsA nephrotoxicity. In chapter 5 we wondered whether differences occur in the ECM accumulation of the renal cortex. Quantitative data of the ECM matrix composition of the cortical interstitium of human allografts suffering from chronic CsA nephrotoxicity or CR were lacking. We chose to study the molecules collagen I, III, and IV, because they are known to accumulate in the renal cortical interstitium during native kidney diseases.[22] The molecules laminin β2 and collagen IV α3 were investigated because an earlier report suggested that *de novo* expression of these molecules at the proximal tubular basement membrane could differentiate CR from chronic CsA nephrotoxicity.[23] We found quantitative and qualitative differences in the ECM composition of kidney allografts suffering from chronic rejecting or chronic CsA nephrotoxicity. The collagens I, III, and IV accumulate in the renal cortical interstitium during CR. The most prominent increases are found for collagen I and III. A similar pattern of matrix deposition was seen in the kidneys of patients who likely had CR but were taking CsA. In the kidneys of patients that suffered from chronic CsA nephrotoxicity, however, only collagens III or IV accumulated significantly.

The regulation of renal interstitial matrix composition *in vivo* is complex and encompasses changes in ECM biosynthesis and degradation. In the normal kidney, the collagens I and III are found in the blood vessels and in the interstitium albeit in small amounts. Collagen IV is a natural component of the glomerular and tubular basement membrane and its α3 chain is normally found in the basement membrane of glomeruli and distal tubules. Laminin is found in vessel walls and in all basement membranes. The β2 chain, however, is only found in glomerular basement membranes and in vessel walls. A few studies reported an increase in collagen III in grafts with chronic dysfunction but did not compare changes induced by CR or chronic CsA nephrotoxicity.[24] A number of *in vitro* studies have addressed the influence of CsA on matrix molecule synthesis by renal cells. One study reported that CsA stimulates the production of pro-collagen I and IV in cultures of murine proximal tubular epithelial cells.[25] In cultured murine renal fibroblasts pro-collagen I synthesis was also stimulated.[26] An increase in the cortical expression of collagens I and IV was found *in vivo* in a rat model of cyclosporine nephrotoxicity.[25] The deposition of collagen III was, however, not assessed in this model. Other data on collagen production were derived from a study on cultured human cells that showed an increase in collagen III production by renal fibroblasts when they were exposed to CsA .[27] Studies done on a monkey renal fibroblast cell line (CV1) showed that CsA stimulates the synthesis of type III collagen by

a pathway that leads to activation of the *COL3A1* promoter and up-regulation of *COL3A1* mRNA.[28] We report an increased deposition of collagen III and IV in the interstitium of the cortex of human renal allografts as a result of chronic CsA nephrotoxicity. In contrast to the results of the study done in rats,[26] we observed no significant increase in collagen I deposition. Although we did not find a correlation between the exposure time to toxic CsA concentrations and the degree of collagen I deposition, we cannot fully exclude that, with time, collagen I may still accumulate during long-term chronic CsA nephrotoxicity. The difference in collagen I staining between the CsA nephrotoxicity and CAN/CR subgroups with mild fibrosis suggests that CR may at least stimulate collagen I accumulation earlier than CsA nephrotoxicity. We also found more expansion of the interstitial space in the CR group compared with the chronic CsA nephrotoxicity group, and also more deposition of collagen type III. The difference in interstitial fibrosis could not be explained by the amount of myofibroblasts present as measured by SMA staining, which marks activated myofibroblasts. We did not find a difference in SMA staining between the CR, CAN, and chronic CsA nephrotoxicity groups. All contained more SMA+ cells than normal. To explain the higher degree of interstitial ECM deposition during CR as compared to chronic CsA nephrotoxicity, one could hypothesize a higher rate of myofibroblasts activity to produce collagen or, alternatively, a lower rate of matrix degradation by tissue metalloproteinases. Data derived from a primate model of chronic cardiac rejection suggest that the inhibition of ECM degradation plays important role in CR, as it was shown that the progression of myocardial fibrosis was associated with increased expression of TIMP-1 and 2, proteins that inhibit matrix degradation.[29]

CsA may also induce tissue remodeling by growth factor and cytokine release. Effects on matrix synthesis and degradation have been found and are reviewed in chapter 2. The results of chapter 6 showed no difference between the chronic rejection group, the CsA toxicity group, and the control group in collagens I and III mRNA levels. The accumulation of collagen in CAN might be explained by an early increase in collagen synthesis that might have taken place before the overt damage seen in the tissues. Alternatively, it might also be that the collagen accumulation is the result of impaired degradation of collagen. Chapter 5 demonstrates that differences in interstitial matrix protein composition might occur during the process of deterioration of allograft function caused by either CR or chronic CsA nephrotoxicity. However, the clinical significance of immunostaining for collagen I, III or IV remains to be established. We did not study follow-up biopsies and do not know exactly know how matrix composition may change over time.

Chapter 6 shows that real-time PCR analysis of the mRNA level of laminin β2 could be a potential new tool to detect chronic CsA nephrotoxicity. However, because the study was small and done on a highly selected population it is too early to recommend this test in

daily clinical practice. The protein laminin β2 is normally found in glomeruli and vessel walls within the kidney and the major increase in cortical laminin β2 mRNA content during chronic CsA nephrotoxicity may be caused by arteriolar vascular damage and subsequent repair. This may explain why the expression of this protein did not increase significantly in the renal cortex of patients with chronic CsA nephrotoxicity. It should also be noted that in chapter 6 the whole renal cortex was studied, whereas in chapter 5 glomeruli and vessels were excluded from the analysis.

In conclusion, long-term CsA therapy has an important effect on the evolution of CAN. Cyclosporine A withdrawal may decrease the number of patients that suffer from late allograft dysfunction. Cyclosporine A appears to be not directly toxic for human proximal tubular epithelial cells. Qualitative and quantitative differences in interstitial matrix composition may occur during the decline of function of a kidney allograft that suffers from either CR or CsA nephrotoxicity. The assessment of cortical laminin β2 mRNA content could be a helpful new diagnostic tool of chronic CsA nephrotoxicity and deserves further study.

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Chapter 8

Samenvatting

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Verlies van nierfunctie na een niertransplantatie is een belangrijk medisch probleem. Bij ongeveer 40-50% van de patiënten die een donornier ontvangt ontstaat na enkele jaren een langzaam progressief verlies van nierfunctie. Deze achteruitgang in functie kan verschillende oorzaken hebben die al of niet specifiek samenhangen met een reactie van het immuunsysteem (afweersysteem) van de ontvanger op de lichaamsvreemde donornier. Wanneer vastgesteld wordt dat de functie van de getransplanteerde nier achteruit gaat, zal meestal een biopt worden genomen (het verkrijgen van een kleine hoeveelheid weefsel met een naald). In dit biopt wordt vaak chronische schade vastgesteld, zich uitend in verbindweefseling en verlies van functionerende cellen van de glomeruli (zeeflichaampjes) en de tubuli (in de nier gelegen kleine afvoerbuisjes van urine). Sinds 1991 worden deze afwijkingen ingedeeld volgens een internationaal aanvaard classificatie systeem het zogenaamde "BANNF-systeem". De toename van bindweefsel naast de tubuli en in de glomeruli, en atrofie (verlies van cellen), wordt binnen dit systeem aangeduid als chronische allograft nefropathie (CAN). De ernst van de interstitiële fibrose (toename van bindweefsel rondom tubuli) en de ernst van atrofie van tubuli bepalen de mate van CAN. Naast aspecifieke verlittekening en verlies van functionerende cellen worden soms meer specifieke afwijkingen in het biopt gezien die kunnen duiden op een bepaalde oorzaak van de achteruitgang van de transplantaatnier. Chronische allograft glomerulopathie (CAG) (een specifieke vorm van ontsteking van de zeeflichaampjes) duidt op een immunologische oorzaak, namelijk chronische afstoting op het niveau van de glomeruli. Deze afwijking wordt echter slechts in een klein percentage van de biopten gevonden. Arteriële intimafibrose (toename van bindweefsel in de slagaders) kan goed passen bij chronische afstoting, maar kan ook een gevolg zijn van een hoge bloeddruk of reeds aanwezig zijn in de nier bij transplantatie. Perifere nodulaire hyaliene degeneratie (PNHD) (bolvormige afzetting van glazig materiaal aan de buitenkant) van de kleinste slagaders is een specifiek kenmerk van chronische schade veroorzaakt door cyclosporine A (CsA) en tacrolimus. Deze medicijnen worden gegeven om afstoting te voorkomen en ontlenen hun specifieke werking aan de remming van het enzym calcineurine. De remming van dit enzym in lymfocyten (bepaald type wit bloedlichaampje) verzwakt de werking van het immuunsysteem. Hoewel PNHD vrij specifiek is voor nierschade als gevolg van deze medicijnen, wordt deze afwijking zeker niet altijd gevonden in biopten van nieren die door deze medicijnen beschadigd zijn geraakt.

Kort geleden is er veel belangstelling ontstaan voor een methode om chronische afstoting vast te stellen door middel van het aantonen van het C4d eiwit, een brokstuk van complementfactor C4 (een eiwit dat onderdeel is van het afweersysteem). De aanwezigheid van dit molecuul in de peritubulaire capillairen (naast de nierbuisjes gelegen haarvaatjes) duidt op lokale complement activatie (actief worden van deze afweereiwitten), en zou kunnen duiden op door antistoffen veroorzaakte weefselchade. Deze antistoffen worden door het immuunsysteem gemaakt en zijn gericht tegen lichaamsvreemde moleculen. Recent is echter ook aangetoond dat activering van het complement systeem zeker niet altijd hoeft te leiden tot weefselschade, omdat cellen zich kunnen aanpassen aan de schadelijke invloeden van complementeiwitten. Op dit moment bestaat er nog geen algemene overeenstemming over de betekenis van de aanwezigheid van het C4d fragment in biopten met CAN. Recent werd ook een sterke associatie vastgesteld tussen CAG en een bij hoge vergroting door middel van elektronen microscopie (EM) waar te nemen sterk toegenomen gelaagdheid van de basaalmembraan (onderlaag) van de peritubulaire capillairen (naast de tubuli gelegen haarvaatjes). De meerwaarde van dit onderzoek voor het stellen van de diagnose chronische afstoting is echter nog niet voldoende bewezen. Overigens is het zo dat de aanwezigheid van markers die duiden op chronische afstoting niet uitsluit dat ook andere processen actief zijn in het beschadigen van de transplantaatnier, zoals toxiciteit van chronische calcineurine remmers.

De onderzoeken in dit proefschrift concentreren zich op de volgende vragen. Wat is de invloed van langdurige blootstelling aan CsA, de oudste en nog steeds veel gebruikte calcineurine remmer, op het verlies van nierfunctie na transplantatie en het ontstaan van CAN? Brengt CsA direct schade toe aan de proximale tubuli (eerste gedeelte van de afvoerbuisjes) van de nier? Is er een verschil in de eiwitsamenstelling van het bindweefsel en in de concentraties van mRNA moleculen die coderen voor bindweefseleiwitten, in nieren met chronische afstoting of chronische cyclosporine toxiciteit?

In Hoofdstuk 2 wordt een overzicht gegeven van wat er in de literatuur bekend is over door CsA veroorzaakte chronische nierschade. Hoewel er in de afgelopen 25 jaar veel onderzoek is verricht, is nog steeds niet duidelijk hoe deze schade precies ontstaat. Ook is het moeilijk de aandoening in het biopt te onderscheiden van chronische afstoting. Eerder onderzoek heeft aangetoond dat meer dan de helft van de patiënten met een langzaam achteruitgaande transplantaatfunctie en CAN baat heeft bij het stoppen of verminderen van de cyclosporine dosering. Dit laat zien dat CsA waarschijnlijk een belangrijke rol speelt bij het late verlies van nierfunctie. Er zijn verschillende hypothesen hoe CsA de nier beschadigt. De belangrijkste zijn: “vernauwing en beschadiging van de kleinste slagaders

van de nier", "directe beschadiging van cellen van de nier", en "directe stimulatie van de vorming van bindweefsel als gevolg van het vrijkomen van groeifactoren in de nier".
In **hoofdstuk 3** is het schadelijk effect van CsA op proximale tubulusepitheel cellen onderzocht. Dit werd gedaan door het effect van CsA te meten aan de hand van het optreden van celdood, via apoptose (geprogrammeerde celdood) of necrose, van proximale tubulusepitheel cellen. CsA werd toegevoegd aan gekweekte menselijke proximale tubulusepitheel cellen (PTEC) en aan dezelfde soort cellen van een varkens cellijn, die de eigenschap heeft eindeloos te kunnen delen ($LLC\text{-}PK_1$-cellen). Een toegenomen celdood kon niet worden aangetoond, ook niet onder condities van zuurstofgebrek of condities die apoptose zouden kunnen bevorderen. Geconcludeerd werd dat dit onderzoek geen steun gaf aan de hypothese dat het toedienen van CsA in de mens op een directe manier schade toebrengt aan proximale tubulusepitheel cellen van de nier.

In hoofdstuk 4 worden de lange termijnresultaten beschreven van een in 1983 gestart onderzoek naar het effect van vervanging van CsA door azathioprine, een afweer onderdrukkend middel dat anders werkt dan CsA en niet toxisch is voor de nier, 3 maanden na niertransplantatie. Kort na introductie van CsA als geneesmiddel in 1978 bleek dat het gebruik van dit middel in plaats van azathioprine een spectaculaire daling gaf van het aantal acute afstotingsreacties, hetgeen resulteerde in een toename van het aantal niertransplantaten dat na 1 jaar nog functioneerde. Een keerzijde van het gebruik van CsA vormde echter de bijwerkingen die bestonden uit hoge bloeddruk, nadelige effecten op diverse vetten in het bloed, nierbeschadiging, jicht, zwelling van het tandvlees en overmatige beharing. Aangezien de kans op een ernstige acute afstoting sterk afneemt na ongeveer 3 maanden na transplantatie werd gepostuleerd, dat vervanging van CsA door het oude middel azathioprine na 3 maanden, van voordeel zou kunnen zijn ter voorkoming van verlies van transplantaatfunctie en ter vermindering van de nare bijwerkingen van CsA. In 1995 werden de resultaten van 8 jaar follow-up gepubliceerd. Er werd toen geen significant verschil gevonden in patiëntsterfte en verlies van transplantaat functie. Wel was er een trend waarneembaar die aangaf dat er na een langere tijd van follow-up mogelijk toch een verschil in transplantaat overleving zou kunnen ontstaan in het voordeel van de azathioprine gebruikers. De overgang naar azathioprine bleek een gunstig effect te hebben op de bloeddruk, het bloed cholesterolgehalte en het voorkomen van jicht. In hoofdstuk 4 worden de resultaten beschreven na een periode van 15 jaar. De sterfte in beide groepen bleek na 15 jaar niet te verschillen maar wel waren er meer patiënten met een nog functionerend niertransplantaat in de azathioprine groep. Er was geen verschil in de transplantaatoverleving wanneer de patiënten werden meegerekend die gedurende het onderzoek van medicatie waren veranderd. Het bleek echter dat veel patiënten in de CsA

groep later toch op azathioprine waren overgegaan omdat, op grond van de uitslag van het biopt, vermoed werd dat zij leden aan door CsA veroorzaakte nierschade. Er werd ook vaker een biopt genomen bij patiënten die CsA gebruikten en ook werd vaker in deze groep de diagnose CAN gesteld. Uit dit onderzoek kon worden geconcludeerd dat langdurige blootstelling aan CsA een belangrijke oorzaak is van verlies van transplantaat functie, en dat het van belang zou kunnen zijn dit middel na verloop van tijd te staken of te vervangen door een ander middel.

In **hoofdstuk 5** vergeleken wij de samenstelling van het bindweefsel in de nierschors van patiënten die ofwel leden aan chronische afstoting, danwel nierbeschadiging hadden opgelopen door langdurige toediening van CsA. Hiervoor werden patiënten geselecteerd met een langzaam achteruitgaande transplantaat functie en door middel van biopsie vastgestelde CAN. Een deel van deze patiënten werd niet behandeld met CsA en kreeg naast prednison azathioprine. Er werd vanuitgegaan dat deze patiënten lijden aan chronische afstoting. Een tweede groep met vermoedelijke chronische afstoting gebruikte wel CsA, maar de afwijkingen in het biopt waren zeer suggestief voor chronische afstoting. Een derde groep had een stabiele transplantaat functie tijdens CsA-gebruik, maar leed aan verlies van transplantaat functie na het gebruik van een nieuw CsA-preparaat, dat beter in het lichaam werd opgenomen. Deze groep werd aangeduid als de groep met chronische schade door CsA. Ter vergelijking werd een controle groep van normale nieren onderzocht. De aanwezigheid van de bindweefseleiwitten collageen I, III en IV, collageen IVα3 en laminine β2 werd onderzocht. Verder werd ook gekeken naar de expressie van het α-smooth muscle actin (SMA), een kenmerkend eiwit van cellen die gespecialiseerd zijn in de vorming van littekenweefsel (myofibroblasten). De eiwitten werden bestudeerd met behulp van een kleuringsmethode die gebruik maakt van een specifieke antistof. De oppervlakte van het gebied dat in het biopt aankleurde werd genomen als maat voor de hoeveelheid eiwit en werd gemeten met behulp van een computerprogramma. Tijdens de computerbewerking werd de aankleuring van bloedvaten en glomeruli (zeeflichaampjes) bewust niet meegerekend. Tevens werd direct microscopisch onderzoek verricht, waarbij de toename van het bindweefsel op een schaal van een tot drie werd gescoord. De toename van bindweefsel was groter in de groep patiënten met chronische afstoting en vermoedelijke chronische afstoting. Hoewel er geen verschil in SMA-kleuring tussen de drie groepen onderling werd gemeten, was de aankleuring voor SMA in alle drie de patiëntengroepen groter dan in de normale controle groep. De chronische afstoting en vermoedelijke chronische afstoting groep hadden wel meer collageen I in het bindweefsel dan de normale controles. Nadere bestudering van een subgroep van patiënten, met slechts een geringe toename van bindweefsel, liet al een verschil in de hoeveelheid collageen I kleuring zien

tussen de chronische afstoting en vermoedelijk chronische afstoting groepen en de groep met schade door CsA. De collagenen III, IV en IVα3 waren toegenomen bij elke groep van patiënten. In de biopten van patiënten met chronische afstoting en vermoedelijke chronische afstoting was het collageen III meer toegenomen dan in de biopten van patiënten met schade door CsA. Aankleuring voor laminine β2 verschilde niet tussen de patiënten groepen en de normale controle groep. Geconcludeerd werd dat structurele schade door CsA vooral leidt tot een toename van de collagenen III en IV en minder door een toename van collageen I. Een toename van collageen I, naast III en IV, wordt vooral gezien bij chronische afstoting .

In **hoofdstuk 6** werden mRNA (messenger ribonucleïnezuur) moleculen gemeten in de nierschors van patiënten die leden aan chronische afstoting of schade door CsA. In de kern van de cel worden de genen, die gecodeerd liggen in het DNA, normaliter afgelezen door polymerases (afleeseiwitten). Hierbij worden mRNA moleculen gevormd, die op hun beurt weer buiten de kern worden afgelezen, en zorgen dat er specifieke eiwitten worden aangemaakt. De patiënten in hoofdstuk 6 werden op klinische en histologische gronden ingedeeld in een groep met chronische, en een groep met chronische CsA niertoxiciteit. Nierbiopten van 19 patiënten met chronische afstoting, 17 patiënten met schade door CsA, en 6 normale transplantatie nieren werden bestudeerd. Gemeten werd het mRNA dat codeerde voor de volgende eiwitten: transforming growth factor β (TGF-β) (een eiwit dat o.a. de vorming van bindweefsel stimuleert), collageen Iα1, IIIα1, IVα3, decorine, fibronectine en laminine β2 (eiwitten van het steun- en bindweefsel). In beide patiënten groepen waren de gemeten mRNA's van collageen IVα3 en fibronectine significant verhoogd ten opzichte van controles. Alleen in de groep met chronische CsA toxiciteit waren de mRNA's van TGF-β en laminine β2 verhoogd. De mRNA's van collageen Iα1, IIIα1, IVα3 en decorine verschilden niet in de drie groepen. Geconcludeerd werd dat het mRNA van TGF-β en laminine β2 sterker is toegenomen in de nierschors van patiënten met schade door CsA dan in de nierschors van patiënten met chronische afstoting. Nader onderzoek naar de waarde van het bepalen van deze mRNA's voor het vaststellen van de diagnose "nierschade door CsA in een transplantaatnier" lijkt gerechtvaardigd.
Kort samengevat zijn de belangrijkste bevindingen van dit proefschrift dat langdurige toediening van CsA de transplantaatfunctie kan aantasten, en dat het van voordeel zou kunnen zijn dit middel na verloop van tijd te staken of te vervangen door een ander middel. De chronische aantasting van de nierfunctie door CsA wordt waarschijnlijk niet veroorzaakt door directe celbeschadiging van de nierbuisjes, zoals eerder wel is gepostuleerd. Het bindweefsel dat gevormd wordt in de nier als gevolg van chronische afstoting lijkt anders van samenstelling te zijn dan het bindweefsel dat gevormd wordt door nierschade als gevolg

van langdurig CsA gebruik. Hierbij wordt er ook een kwantitatief verschil gevonden in mRNA moleculen die coderen voor de eiwitten TGF-β en laminine β2. Meting van met name de hoeveelheid laminine β2 mRNA in de transplantaatnier zou informatie kunnen verschaffen over de aanwezigheid van CsA toxiciteit.

Nawoord

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 27-12-1960 te Hillegom. In 1979 behaalde hij het diploma Gymnasium β aan het Triniteitslyceum te Haarlem. In hetzelfde jaar werd aangevangen met de studie Geneeskunde aan de Universiteit van Amsterdam. Het doctoraal examen werd in 1984 cum laude afgelegd en in 1987 werd het artsexamen behaald. Van 1987 tot 1989 was hij werkzaam als arts-assistent Inwendige Geneeskunde in het Slotervaart Ziekenhuis te Amsterdam. In 1989 werd in dit ziekenhuis gestart met de opleiding Inwendige Geneeskunde (opleider Prof. Dr. L.W. Statius van Eps). In 1993 werd de opleiding voortgezet in het Academisch Medisch Centrum te Amsterdam (opleider Prof. Dr. L. Arisz). In 1995 werd hij als internist ingeschreven in het Specialisten Register. Van 1995 tot 1996 was hij werkzaam als chef de clinique op de afdeling afdeling Maag-, Darm- en Leverziekten van het Slotervaartziekenhuis te Amsterdam en van 1996 tot 1998 als internist in het Onze Lieve Vrouwe Gasthuis te Amsterdam. Van 1998 tot 2002 was hij verbonden aan de Vakgroep Nierziekten van het Leids Universitair Medisch Centrum (Afdelingshoofd achtereenvolgens Prof. Dr. L.A. van Es en Prof. Dr. L.C. Paul), waar de opleiding tot nefroloog werd afgerond in 2002. In deze periode werd het onderzoek verricht, waarvan de resultaten in dit proefschrift zijn beschreven. Vanaf 1 oktober 2002 is de auteur werkzaam in het Amphia Ziekenhuis te Breda als internist-nefroloog binnen de maatschap inwendige geneeskunde en gastro-enterologie.

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